(54) Title: CONFORMATIONALLY CONSTRAINED PARATHYROID HORMONE (PTH) ANALOGS

(57) Abstract: The present invention relates to conformationally constrained parathyroid hormone (PTH) analogs, and methods of preparing and using the PTH analogs. The invention provides novel PTH polypeptide derivatives containing amino acid substitutions at selected positions in the polypeptides. The invention provides derivatives of PTH (1-34), PTH(1-21), PTH(1-20), PTH(1-19), PTH(1-18), PTH(1-17), PTH(1-16), PTH(1-15), PTH(1-14), PTH(1-13), PTH(1-12), PTH(1-11) and PTH(1-1 0) polypeptides, wherein at least one residue in each polypeptide is a helix, preferably an a-helix, stabilizing residue. The invention also provides methods of making such peptides. Further, the invention encompasses compositions and methods for use in limiting undesired bone loss in a vertebrate at risk of such bone loss, in treating conditions that are characterized by undesired bone loss or by the need for bone growth, e.g. in treating fractures or cartilage disorders and for raising cAMP levels in cells where deemed necessary.

# Conformationally Constrained Parathyroid Hormone (PTH) Analogs

## Background of the Invention

### Field of the Invention

5          The present invention relates to conformationally constrained parathyroid hormone (PTH) analogs, and methods of preparing and using the PTH analogs.

### Background Art

#### Parathyroid hormone

Parathyroid hormone (PTH), an 84 amino acid peptide, is the principal
10       regulator of ionized blood calcium in the human body (Kronenberg, H.M., *et al.*,
In *Handbook of Experimental Pharmacology*, Mundy, G.R., and Martin, T.J.,
(eds), pp. 185-201, Springer-Verlag, Heidelberg (1993)). Regulation of calcium
concentration is necessary for the normal function of the gastrointestinal, skeletal,
neurologic, neuromuscular, and cardiovascular systems. PTH synthesis and
15       release are controlled principally by the serum calcium level; a low level
stimulates and a high level suppresses both hormone synthesis and release. PTH,
in turn, maintains the serum calcium level by directly or indirectly promoting
calcium entry into the blood at three sites of calcium exchange: gut, bone, and
kidney. PTH contributes to net gastrointestinal absorption of calcium by favoring
20       the renal synthesis of the active form of vitamin D. PTH promotes calcium
resorption from bone indirectly by stimulating differentiation of the bone-
resorbing cells, osteoclasts. It also mediates at least three main effects on the
kidney: stimulation of tubular calcium reabsorption, enhancement of phosphate
clearance, and promotion of an increase in the enzyme that completes synthesis
25       of the active form of vitamin D. PTH is thought to exert these effects primarily
through receptor-mediated activation of adenylate cyclase and/or
phospholipase C.

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Disruption of calcium homeostasis may produce many clinical disorders (*e.g.*, severe bone disease, anemia, renal impairment, ulcers, myopathy, and neuropathy) and usually results from conditions that produce an alteration in the level of parathyroid hormone. Hypercalcemia is a condition that is characterized by an elevation in the serum calcium level. It is often associated with primary hyperparathyroidism in which an excess of PTH production occurs as a result of a parathyroid gland lesion (*e.g.*, adenoma, hyperplasia, or carcinoma). Another type of hypercalcemia, humoral hypercalcemia of malignancy (HHM) is the most common paraneoplastic syndrome. It appears to result in most instances from the production by tumors (*e.g.*, squamous, renal, ovarian, or bladder carcinomas) of a class of protein hormone which shares amino acid homology with PTH. These PTH-related proteins (PTHrP) appear to mimic certain of the renal and skeletal actions of PTH and are believed to interact with the PTH receptor in these tissues.

### Osteoporosis

Osteoporosis is a potentially crippling skeletal disease observed in a substantial portion of the senior adult population, in pregnant women and even in juveniles. The term osteoporosis refers to a heterogeneous group of disorders. Clinically, osteoporosis is separated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at menopause, while osteoporosis type II is associated with advancing age. Patients with osteoporosis would benefit from new therapies designed to promote fracture repair, or from therapies designed to prevent or lessen the fractures associated with the disease.

The disease is marked by diminished bone mass, decreased bone mineral density (BMD), decreased bone strength and an increased risk of bone fracture. At present, there is no effective cure for osteoporosis, though estrogen, calcitonin and the bisphosphonates, etidronate and alendronate are used to treat the disease with varying levels of success. These agents act to decrease bone resorption. Since parathyroid hormone regulates blood calcium and the phosphate levels, and

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has potent anabolic (bone-forming) effects on the skeleton, in animals (Shen, V., *et al., Calcif. Tissue Int. 50*:214-220 (1992); Whitefild, J.F., *et al., Calcif. Tissue Int. 56*:227-231 (1995) and Whitfield, J.F., *et al., Calcif. Tissue Int. 60*:26-29 (1997)) and humans (Slovik, D.M., *et al., J. Bone Miner. Res. 1*:377-381 (1986);

5        Dempster, D.W., *et al., Endocr. Rev. 14*:690-709 (1993) and Dempster, D.W., *et al., Endocr. Rev. 15*:261 (1994)) when administered intermittently, PTH, or PTH derivatives, are prime candidates for new and effective therapies for osteoporosis.


### *PTH Derivatives*


         PTH derivatives include polypeptides that have amino acid substitutions

10       or are truncated relative to the full length molecule. Both a 14 and a 34 amino acid amino-terminal truncated form of PTH, as well as a C-terminal truncated form have been studied. Additionally, amino acid substitutions within the truncated polypeptides have also been investigated.

         Synthetic PTH(1-34) exhibits full bioactivity in most cell-based assay

15       systems, has potent anabolic effects on bone mass in animals and has recently been shown to reduce the risk of bone fracture in postmenopausal osteoporotic women (Neer, R.M., *et al., N.E.J.M. 344*:1434-1441 (2001); Dempster, D.W., *et al., Endocr Rev 14*:690-709 (1993)). PTH acts on the PTH/PTHrP receptor (P1R), a class II G protein-coupled heptahelical receptor that couples to the

20       adenylyl cyclase/CAMP and phospolipase C/inositol phosphate (IP) signaling pathway (Rippner, H., *et al., Science 254*:1024-1026 (1991)). Deletion analysis studies have shown that the amino-terminal residues of PTH play a crucial role in stimulating the P1R to activate the cAMP and IP signaling pathways (Tregear, G.W., *et al., Endocrinology 93*:1349-1353 (1973); Takasu, H., *et al.,

25       Biochemistry 38*:13453-13460 (1999)). Crosslinking and receptor mutagenesis studies have indicated that residues in the amino-terminal portion of PTH interact with the extracellular loops and extracellular ends of the seven transmembrane helices, which reside within the juxtamembrane region of the receptor (Bergwitz, C., *et al., J. Biol. Chem. 271*:26469-26472 (1996); Hoare, S.R.J., *et al., J. Biol.

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*Chem 276*:7741-7753 (2001); Behar, V., *et al.*, *J. Biol. Chem. 275*:9-17 (1999); Shimizu, M., *et al.*, *J. Biol. Chem. 275*:19456-19460 (2000); Luck, M.D., *et al.*, *Molecular Endocrinology 13*:670-680 (1999)).

## *Brief Summary of the Invention*

5      The invention provides novel PTH polypeptide derivatives containing amino acid substitutions at selected positions in the polypeptides. The derivatives function as full, or nearly full, agonists of the PTH-1 receptor. Because of their unique properties, these polypeptides have a utility as drugs for treating human diseases of the skeleton, such as osteoporosis.

10     The invention provides derivatives of PTH(1-21), PTH(1-20), PTH(1-19), PTH(1-18), PTH(1-17), PTH(1-16), PTH(1-15), PTH(1-14), PTH(1-13), PTH(1-12), PTH(1-11) and PTH(1-10) polypeptides, wherein at least one residue in each polypeptide is a helix, preferably an $\alpha$-helix, stabilizing residue. The invention also provides methods of making such peptides. Further, the invention

15     encompasses compositions and methods for use in limiting undesired bone loss in a vertebrate at risk of such bone loss, in treating conditions that are characterized by undesired bone loss or by the need for bone growth, e.g. in treating fractures or cartilage disorders and for raising cAMP levels in cells where deemed necessary.

20     In one aspect, the invention is directed to a biologically active peptide consisting essentially of $X_{01}ValX_{02}GluIleGlnLeuMetHisX_{03}X_{04}X_{05}X_{06}X_{07}$ (SEQ ID NO: 1), wherein $X_{01}$ is an $\alpha$-helix-stabilizing residue, desaminoGly, desaminoSer or desaminoAla; $X_{02}$ is an $\alpha$-helix-stabilizing residue, Ala, or Ser; $X_{03}$ is Ala, Gln or Asn; $X_{04}$ is Arg, Har or Leu; $X_{05}$ is an $\alpha$-helix-stabilizing

25     residue, Ala or Gly; $X_{06}$ is an $\alpha$-helix-stabilizing residue or Lys; and $X_{07}$ is an $\alpha$-helix-stabilizing residue, Trp or His; and wherein at least one of $X_{01}, X_{02}, X_{03}, X_{04}, X_{05}, X_{06}$ or $X_{07}$ is an $\alpha$-helix-stabilizing residue.

In another aspect, the invention relates to SEQ ID NO: 1, wherein the $\alpha$-helix-stabilizing amino acid is selected from the group consisting of Aib, ACPC

(1-aminocyclopropylcarboxylic acid), DEG (diethylglycine) and 1-aminocyclopentanecarboxylic acid. In another aspect, the invention relates to SEQ ID NO: 1, wherein the α-helix-stabilizing amino acid is Aib.

The invention is further drawn to fragments of the peptide of SEQ ID NO: 1, in particular $X_{01}ValX_{02}GluIleGlnLeuMetHisX_{03}X_{04}X_{05}X_{06}$ (SEQ ID NO: 12), $X_{01}ValX_{02}GluIleGlnLeuMetHisX_{03}X_{04}X_{05}$ (SEQ ID NO: 13), $X_{01}ValX_{02}GluIleGlnLeuMetHisX_{03}X_{04}$ (SEQ ID NO: 14) and $X_{01}ValX_{02}GluIleGlnLeuMetHisX_{03}$ (SEQ ID NO: 15). The invention further encompasses pharmaceutically acceptable salts of the above-described peptides, and N- or C-derivatives of the peptides. A preferable embodiment of the invention is drawn to any of the above recited polypeptides, wherein the polypeptide contains a C-terminal amide.

In addition, the invention is drawn to a biologically active polypeptide consisting essentially of AibValAibGluIleGlnLeuNleHisGlnHarAlaLysTrpLeu-AlaSerValArgArgTyr (SEQ ID NO. 8); fragments thereof, containing amino acids 1-20, 1-19, 1-18, 1-17, 1-16 or 1-15; pharmaceutically acceptable salts thereof; or N- or C- derivatives thereof.

The invention is further drawn to any of the above polypeptides labeled with a label selected from the group consisting of: a radiolabel, a flourescent label, a bioluminescent label, or a chemiluminescent label. In a preferable embodiment the radiolabel is $^{125}I$ or $^{99m}Tc$.

Preferred embodiments of the biologically active peptide include: AibValSerGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 2); desamino-AlaValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 3); desamino-SerValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 4); desamino-GlyValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 5); AibValAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 6); AibValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 7); AibValAlaGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 9); AlaValAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 10); SerValAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 11); and

AibValAibGluIleGlnLeuMetHisGlnHar (SEQ ID NO. 16). It is contemplated that fragments of the above mentioned peptides, containing amino acids 1-10, 1-11, 1-12 or 1-13, are also embodiments of the present invention. The invention further encompasses pharmaceutically acceptable salts of the above-described peptides, and N- or C-derivatives of the peptides.

Other constrained amino acids that are substituted for Aib are ACPC (1-aminocyclopropylcarboxylic acid), DEG (diethylglycine) and 1-aminocyclopentanecarboxylic acid.

In accordance with yet a further aspect of the invention, this invention also provides pharmaceutical compositions comprising a PTH derivative and a pharmaceutically acceptable excipient and/or a pharmaceutically acceptable solution such as saline or a physiologically buffered solution.

This invention also provides a method for treating mammalian conditions characterized by decreases in bone mass, which method comprises administering to a subject in need thereof an effective bone mass-increasing amount of a biologically active PTH polypeptide. A preferable embodiment of the invention is drawn to conditions such as osteoporosis. The types of osteoporosis include, but are not limited to old age osteoporosis and postmenopausal osteoporosis. Additional preferable embodiments include using an effective amounts of the polypeptide of about 0.01 µg/kg/day to about 1.0 µg/kg/day wherein the polypeptide is administered parenterally, subcutaneously or by nasal insufflation.

In accordance with yet a further aspect of the invention, this invention also provides a method for determining rates of bone reformation, bone resorption and/or bone remodeling comprising administering to a patient an effective amount of a labeled PTH polypeptide, such as for example, SEQ ID NO: 1 or a derivatives thereof and determining the uptake of the peptide into the bone of the patient. The peptide is labeled with a label selected from the group consisting of: radiolabel, flourescent label, bioluminescent label, or chemiluminescent label. An example of a suitable radiolabel is $^{99m}$Tc.

The invention is further related to a method of increasing cAMP in a mammalian cell having PTH-1 receptors, the method comprising contacting the

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cell with a sufficient amount of the polypeptide of the invention to increase cAMP.

The invention also provides derivatives of rat PTH(1-34) (rPTH(1-34)) given by AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAlaSerValGluArg MetGlnTrpLeuArgLysLysLeuGlnAspValHisAsnPhe (SEQ ID NO: 30), and of human PTH(1-34) (hPTH(1-34)) given by SerValSerGluIleGlnLeuMetHisAsn LeuGlyLysHisLeuAsnSerMetGluArgValGluTrpLeuArgLysLysLeuGlnAspVal HisAsnPhe (SEQ ID NO: 31).

In another aspect, the invention relates to a biologically active peptide consisting essentially of the formula $X_{01}ValX_{02}GluIleGlnLeuX_{03}HisX_{04}X_{05}X_{06}X_{07}$ $X_{08}LeuX_{09}SerX_{10}X_{11}ArgX_{12}X_{13}TrpLeuArgLysLysLeuGlnAspValHisAsnX_{14}$ (SEQ ID NO: 19) wherein $X_{01}$ is an α-helix-stabilizing residue, desaminoGly, desaminoSer or desaminoAla; $X_{02}$ is an α-helix-stabilizing residue, Ala, or Ser; $X_{03}$ is Met or Nle; $X_{04}$ is Ala, Gln or Asn; $X_{05}$ is Arg, Har or Leu; $X_{06}$ is an α-helix-stabilizing residue, Ala or Gly; $X_{07}$ is an α-helix-stabilizing residue or Lys; $X_{08}$ is an α-helix-stabilizing residue, Trp or His; $X_{09}$ is Ala or Asn; $X_{10}$ is Met or Val; $X_{11}$ is Arg or Glu; $X_{12}$ is Met or Val; $X_{13}$ is Gln or Glu; $X_{14}$ is Tyr or Phe; and wherein at least one of $X_{01}$, $X_{02}$, $X_{06}$, $X_{07,}$ or $X_{08}$ is an α-helix-stabilizing residue. The invention also relates to fragments thereof, containing amino acids 1-33, 1-32, 1-31, 1-30, 1-29, 1-28, 1-27, 1-26, 1-25, 1-24, 1-23, 1-22, 1-21, 1-20, 1-19, 1-18, 1-17, 1-16, 1-15, 1-14, 1-13, 1-12, or 1-11. The invention also relates to pharmaceutically acceptable salts and N- or C- derivatives of SEQ ID NO: 19 or the above described fragments.

In another aspect, the invention relates to SEQ ID NO: 19, wherein the α-helix-stabilizing amino acid is selected from the group consisting of Aib, ACPC (1-aminocyclopropylcarboxylic acid), DEG (diethylglycine) and 1-aminocyclopentanecarboxylic acid. In another aspect, the invention relates to SEQ ID NO: 19, wherein the α-helix-stabilizing amino acid is Aib.

In another aspect, the invention relates specifically to the following peptides: $AibValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuX_{09}SerX_{10}X_{11}Arg$

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$X_{12}X_{13}$TrpLeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO. 20); desaminoAlaValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis Leu$X_{09}$Ser$X_{10}X_{11}$Arg$X_{12}X_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO. 21); desaminoSerValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis Leu$X_{09}$Ser$X_{10}X_{11}$Arg$X_{12}X_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO. 22); desaminoGlyValAibGluIleGlnLeuMetHisAsnLeuGlyLysHisLeu $X_{09}$Ser$X_{10}X_{11}$Arg$X_{12}X_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO. 23); AibValAibGluIleGlnLeuMetHisGlnHarGlyLysTrpLeu$X_{09}$Ser $X_{10}X_{11}$Arg$X_{12}X_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO. 24); AibValAibGluIleGlnLeuMetHisAsnLeuGlyLysHisLeu$X_{09}$Ser $X_{10}X_{11}$Arg$X_{12}X_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO. 25); AibValAlaGluIleGlnLeuMetHisGlnHarAlaLysTrpLeu$X_{09}$Ser$X_{10}X_{11}$Arg$X_{12}X_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO. 26); AlaValAibGluIleGlnLeuMetHisGlnHarAlaLysTrpLeu$X_{09}$Ser$X_{10}X_{11}$Arg$X_{12}X_{13}$ TrpLeuArgLysLysLeuGlnAspValHisAsn $X_{14}$ (SEQ ID NO. 27); and SerValAibGluIleGlnLeuMetHisGlnHarAlaLysTrpLeu $X_{09}$Ser $X_{10}X_{11}$Arg$X_{12}X_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO. 28). $X_{09}$, $X_{10}$, $X_{11}$, $X_{12}$, $X_{13}$ and $X_{14}$ have the same meaning as defined for SEQ ID NO: 19. The invention also relates to pharmaceutically acceptable salts or N- or C- derivatives of the above peptides.

The invention also relates to a biologically active peptide consisting essentially of the formula AibValAibGluIleGlnLeuNleHisGlnHarAlaLysTrpLeu AlaSerValArgArg$X_{12}X_{13}$TrpLeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO: 29) wherein $X_{12}$ is Met or Val; $X_{13}$ is Gln or Glu; and $X_{14}$ is Tyr or Phe. The invention also relates to pharmaceutically acceptable salts or N- or C- derivatives of SEQ ID NO: 29. The invention also relates to fragments thereof, containing amino acids 1-33, 1-32, 1-31, 1-30, 1-29, 1-28, 1-27, 1-26, 1-25, 1-24, 1-23, 1-22, 1-21, 1-20, 1-19, 1-18, 1-17, 1-16, 1-15, 1-14, 1-13, 1-12, or 1-11.

In another aspect of the invention, SEQ ID NO: 19, SEQ ID NO: 29 or any of the above peptides are labeled. In another aspect of the invention, SEQ ID NO: 19, SEQ ID NO: 29 or any of the above peptides are labeled with a

fluorescent label, a chemiluminescent label; a bioluminescent label; a radioactive
label; $^{125}$I; or $^{99m}$Tc.

In another aspect, the invention is directed to a pharmaceutical
composition comprising the biologically active peptide SEQ ID NO: 19, SEQ ID
NO: 29 or any of the above peptides, and a pharmaceutically acceptable carrier.

In another aspcet, the invention is directed to a method for treating
mammalian conditions characterized by decreases in bone mass, the method
comprising administering to a subject in need thereof an effective bone mass-
increasing amount of a biologically active peptide of SEQ ID NO: 19, SEQ ID
NO: 29 or any of the above peptides.

In another aspcet, the invention is directed to a method for treating
mammalian conditions characterized by decreases in bone mass, the method
comprising administering to a subject in need thereof an effective bone mass-
increasing amount of a composition comprising a biologically active peptide of
SEQ ID NO: 19, SEQ ID NO: 29 or any of the above peptides and a
pharmaceutically acceptable carrier.

In another aspect of the invention, the condition to be treated is
osteoporosis, old age osteoporosis, or post-menopausal osteoporosis. In another
aspect of the invention, the effective amount of SEQ ID NO: 19, SEQ ID NO: 29
or any of the above peptides for increasing bone mass is from about 0.01
μg/kg/day to about 1.0 μg/kg/day. In another aspect of the invention, the method
of administration is parenteral, subcutaneous or nasal insufflation.

In another aspcet, the invention is directed to a method for determining
rates of bone reformation, bone resorption and/or bone remodeling comprising
administering to a patient an effective amount of SEQ ID NO: 19, SEQ ID NO:
29 or any of the above peptides and determining the uptake of the peptide into the
bone of the patient.

In another aspcet, the invention is directed to a method of making SEQ
ID NO: 19, SEQ ID NO: 29 or any of the above peptides, wherein the peptide is
synthesized by solid phase synthesis.

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In another aspect, the invention is directed to a method of making SEQ ID NO: 19, SEQ ID NO: 29 or any of the above peptides, wherein the peptide is protected by FMOC.

## *Brief Description of The Figures*

FIG. 1. Aib-scan of a modified PTH(1-14) analog in HKRK-B28 cells. The peptide $[Ala^{3,12},Gln^{10},Har^{11},Trp^{14}]PTH(1-14)$ amide $\{[M]PTH(1-14)\}$, and derivatives of that peptide containing a single Aib substitution at one of each residue position, were evaluated for the capacity to stimulate intracellular cAMP accumulation in HKRK-B28 cells. The peptides with substitutions at position 1-7 are shown in panel A, and those with substitutions at position 8-9 are shown in B. Shown are combined data (mean±S.E.M.) from 3 to 10 experiments, each performed in duplicate. Symbols are defined in the key.

FIG. 2. cAMP-signaling and binding properties of PTH analogs in HKRK-B28 cells. Peptides were evaluated in HKRK-B28 cells for the capacity to stimulate intracellular cAMP accumulation (A) and the capacity to inhibit binding of $^{125}I$-$[M]PTH(1-21)$ (B). Shown are combined data (mean±S.E.M.) from 3 or 4 experiments, each performed in duplicate. Peptides and corresponding symbols are identified in the key.

FIG. 3. Signaling and binding properties of PTH analogs in COS-7 cells expressing an N-terminally truncated P1R. COS-7 cells were transiently transfectd with P1R-delNt, a truncated P1R which is deleted for most of the amino-terminal extracellular domain, and subsequently used to evaluate the capacities of the indicated PTH analogs to stimulate intracellular cAMP accumulation (A); stimulate formation of $^{3}H$-inositol phosphates $(IP^{1}+IP^{2}+IP^{3})$ (B); and inhibit the binding of $^{125}I$-$[Aib^{1,3},M]PTH(1-21)$ (C). Each curve shows data combined (mean±S.E.M.) from 3 to 6 experiments, each performed in duplicate. The mean basal level of $^{3}H$-inositol phosphates (2,929±877 cpm/well) is indicated by the dashed line. Peptides and corresponding symbols are identified in the key.

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FIG. 4. cAMP-signaling properties of PTH analogs in SaOS-2 cells. The peptides, PTH(1-34), native PTH(1-14), [M]PTH(1-14) and [Aib$^{1,3}$,M]PTH(1-14) were evaluated in the human osteosarcoma-derived cell line SaOS-2 for the capacity to stimulate intracellular cAMP accumulation. Shown are combined data (mean±S.E.M.) from 3 or 4 experiments, each performed in duplicate. Symbols are defined in the Key.

FIG. 5. Effect of PTH analogs on bone mineralization in embryonic mouse metatarsals. Cartilaginous metatarsal bone rudiments were excised from E15.5 mouse embryos and transferred to tissue culture plates containing serum-free media. Added to the samples for 48 h were vehicle: (A); PTH(1-34) (0.1μM) (B); [Aib$^{1,3}$,M]PTH(1-14) (1 μM) (C) or native PTH(1-14) (2 μM) (D). Samples were explanted and incubated at 37°C for a total of 64 h; peptide or vehicle were added at 16 h and again at 24 h. At the end of the incubation, the samples were fixed, sectioned and directly visualized under white light using a dissecting scope. In the vehicle- and native PTH(1-14)-treated samples mineralization can be detected as dark material at the center of the bone rudiment. Both PTH(1-34) and [Aib$^{1,3}$,M]PTH(1-14) inhibited mineralization. Shown are data from a single experiment, comparable results were obtained in three other replicate experiments.

FIG. 6. Circular Dichroism Spectroscopy. Spectra were recorded for the indicated N-terminal PTH oligopeptides, each at 20 μM, in 50 nM sodium phosphate buffer, pH 7.4 containing 20% 2,2,2,-trifluoroethanol. The negative extrema in the spectra at ~209 nM and ~222 nM, and the positive extrema at ~192 nM, which are more apparent in the Aib-containing PTH analogs, as compared to the non-Aib-containing peptides, are indicative of helical content.

FIG. 7. Signaling and binding properties of hPTH(1-34) analogs in COS-7 cells expressing wildtype P1R (hP1R-WT, FIG. 7A) and N-terminally truncated P1R (hP1R-delNT, FIG. 7B). The COS-7 cells were used to evaluate the capacities of the indicated PTH analogs to stimulate intracellular cAMP accumulation. Cells expressing hP1R-delNT were prepared as described above. Peptides and corresponding symbols are identified in the key.

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# Detailed Description of The Invention

## Definitions

Amino Acid Sequences: The amino acid sequences in this application use either the single letter or three letter designations for the amino acids. These designations are well known to one of skill in the art and can be found in numerous readily available references, such as for example in *Cooper, G.M., The Cell* 1997, ASM Press, Washington, D.C. or Ausubel et al., *Current Protocols in Molecular Biology,* 1994 . Where substitutions in a sequence are referred to, for example, as Ser-3 -->Ala or [Ala³]peptide, this means that the serine in the third position from the N-terminal end of the polypeptide is replaced with another amino acid, Alanine in this instance.

In the present application [M]PTH(1-14) is defined as [Ala$^{3,12}$,Gln$^{10}$,Har$^{11}$,Trp$^{14}$]PTH(1-14)amide. [M]PTH(1-21) is defined as [Ala$^{3,12}$,Nle$^8$,Gln$^{10}$,Har$^{11}$,Trp$^{14}$,Arg$^{19}$,Tyr$^{21}$]PTH(1-21)amide. [M]PTH(1-11) is defined as [Ala$^3$,Gln$^{10}$,Har$^{11}$]PTH(1-11)amide.

In the present application, "Aib" refers to α-aminoisobutyric acid; "Har" refers to homoarginine; "Nle" refers to norleucine; and other amino acids are in either the conventional one- or three-letter codes.

Biological Activity of the Protein: This expression refers to any biological activity of the polypeptide. Examples of these activities include, but are not limited to metabolic or physiologic function of compounds of SEQ ID NO: 1 or SEQ ID NO: 8 or derivatives thereof, including similar activities or improved activities, or those activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of the above-described compounds.

Derivative or Functional Derivative: The term "derivative" or "functional derivative" is intended to include "variants," the "derivatives," or "chemical derivatives" of the PTH molecule. A "variant" of a molecule such as for example, a compound of SEQ ID NO: 1 or derivative thereof is meant to refer to a

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molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule such as for example, a compound of SEQ ID NO: 1 or derivative thereof is meant to refer to a non-natural molecule substantially similar to either the SEQ ID NO: 1 molecules or fragments thereof.

5          PTH derivatives contain changes in the polypeptide relative to the native PTH polypeptide of the same size. The sequence of the native PTH(1-14) polypeptide is the first fourteen amino acids of SEQ. ID NO: 17 (human PTH (1-21)) or SEQ. ID NO: 18 (rat PTH (1-21)). A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both

10         molecules is substantially the same, and if both molecules possess a similar biological activity. Thus, two molecules that possess a similar activity, may be considered variants, derivatives, or analogs as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. PTH derivatives,

15         however, need not have substantially similar biological activity to the native molecule. In some instances PTH derivatives have substantially different activity than the native PTH. For example, a derivative may be either an antagonist or an agonist of the PTH receptor.

           As used herein, a molecule is said to be a "chemical derivative" of another

20         molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Examples of moieties capable of mediating such effects are disclosed in

25         *Remington's Pharmaceutical Sciences* (1980) and will be apparent to those of ordinary skill in the art.

           Fragment: A "fragment" of a molecule such as for example, SEQ ID NO: 1 or derivative thereof is meant to refer to any polypeptide subset of these molecules.

30         Fusion protein: By the term "fusion protein" is intended a fused protein comprising compounds such as for example, SEQ ID NO: 1 or derivatives

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thereof, either with or without a "selective cleavage site" linked at its N-terminus, which is in turn linked to an additional amino acid leader polypeptide sequence.

Polypeptide: Polypeptide and peptide are used interchangeably . The term polypeptide refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids and include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in the research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications.

Polypeptides may be branched and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translational modifications or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. *See,* for instance, *Proteins-Structure and Molecular Properties*, 2nd Ed., T. E.

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Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and nonprotein cofactors", *Methods in Enzymol. 182*:626-646 (1990) and Rattan *et al.*, "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci 663*:48-62 (1992).

### *PTH Analogs -- Structural and Functional Properties*

α-aminoisobutyric acid (Aib) was introduced into short N-terminal PTH peptide analogs. The numerous NMR studies of PTH(1-34) analogs, performed in a variety of polar or non-polar solvents, have generally indicated two domains of secondary structure: a stable C-terminal helix extending approximately from Ser-17 to Val-31, and a shorter and less stable amino-terminal helix, extending variably from Ser-3 to Lys-13, the two domain being connected by a bend or turn region (Marx, U.C., *et al., Biochem. Biophys. Res. Commun. 267*:213-220 (2000); Chen, Z., *et al., Biochemistry 39*:12766-12777 (2000); Marx, U.C., *et al., J. Biol. Chem. 270*:15194-15202 (1995); Marx, U.C., *et al., J. Biol. Chem. 273*:4308-4316 (1998); Pellegrini, M., *et al., Biochemistry 37*:12737-12743 (1998); Gronwald, W., *et al., Biol. Chem. Hoppe Seyler 377*:175-186 (1996); Barden, J.A., and Kemp, B.E., *Biochemistry 32*:7126-7132 (1993)). The recent crystallographic study of PTH(1-34) indicated a continuous α-helix extending from Ser-3 to His-32 and containing only a slight 15° bend at the midsection. However, NMR data indicates that the N-terminal α-helix is relatively weak. Helix-stabilizing modifications, such as the introduction of Aib residues, offer significant benefits in terms of peptide potency, and result in short peptides ($\leq 14$ amino acids) with activity comparable to PTH(1-34).

Described herein are novel "minimized" variants of PTH that are small enough to be deliverable by simple non-injection methods. The variants of the present invention contain substitutions in the first 14 amino acids of the

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polypeptide. The new polypeptides correspond to the 1-21, 1-20, 1-19, 1-18, 1-17, 1-16, 1-15, 1-14, 1-13, 1-12, 1-11, and 1-10 amino acid sequence of the mature PTH polypeptide. The shorter variants (≤ PTH1-14) have a molecular weight of less than 2,000 daltons.

5          The primary amino acid sequence of the native human PTH(1-21) peptide (N-terminus to C-terminus) is SerValSerGluIleGlnLeuMetHisAsnLeuGlyLys-HisLeuAsnSerMetGluArgVal (SEQ ID NO: 17), whereas the primary sequence of the native rat PTH (1-21) is AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLys-HisLeuAlaSerValGluArgMet (SEQ ID NO. 18).

10         As protein products, compounds described herein are amenable to production by the techniques of solution- or solid-phase peptide synthesis. The solid phase peptide synthesis technique, in particular, has been successfully applied in the production of human PTH and can be used for the production of these compounds (for guidance, *see* Kimura *et al., supra*, and see Fairwell *et al., Biochem. 22*:2691 (1983)). Success with producing human PTH on a relatively large scale has been reported by Goud *et al., in J. Bone Min. Res. 6(8)*:781 (1991). The synthetic peptide synthesis approach generally entails the use of automated synthesizers and appropriate resin as solid phase, to which is attached the C-terminal amino acid of the desired compounds of SEQ ID NO: 1 or derivatives thereof. Extension of the peptide in the N-terminal direction is then achieved by successively coupling a suitably protected form of the next desired amino acid, using either FMOC- or BOC-based chemical protocols typically, until synthesis is complete. Protecting groups are then cleaved from the peptide, usually simultaneously with cleavage of peptide from the resin, and the peptide is then isolated and purified using conventional techniques, such as by reversed phase HPLC using acetonitrile as solvent and tri-fluoroacetic acid as ion-pairing agent. Such procedures are generally described in numerous publications and reference may be made, for example, to Stewart and Young, "Solid Phase Peptide Synthesis," 2nd Edition, Pierce Chemical Company, Rockford, IL (1984). It will be appreciated that the peptide synthesis approach is required for production of

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such as for example, SEQ ID NO: 1 and derivatives thereof which incorporate amino acids that are not genetically encoded, such as Aib.

In accordance with another aspect of the present invention, substituents are attached to the free amine of the N-terminal amino acid of compounds of the present invention standard methods known in the art. For example, alkyl groups, e.g., $C_{1-12}$ alkyl, are attached using reductive alkylation. Hydroxyalkyl groups, e.g. $C_{1-12}$ hydroxyalkyl, are also attached using reductive alkylation wherein the free hydroxy group is protected with a t-butyl ester. Acyl groups, e.g., $COE_1$, are attached by coupling the free acid, e.g., $E_1COOH$, to the free amino of the $N$-terminal amino acid. Additionally, possible chemical modifications of the C-terminal end of the polypeptide are encompassed within the scope of the invention. These modifications may modify binding affinity to the receptor.

Also contemplated within the scope of this invention are those compounds such as for example, SEQ ID NO:1 and derivatives thereof with altered secondary or tertiary structure, and/or altered stability, which still retain biological activity. Such derivatives might be achieved through lactam cyclization, disulfide bonds, or other means known to a person of ordinary skill in the art.

## *Utility and Administration of Compounds of the Invention*

Compounds of the invention or derivatives thereof have multiple uses. These include, *inter alia,* agonists or antagonists of the PTH receptor, prevention and treatment of a variety of mammalian conditions manifested by loss of bone mass, diagnostic probes, antigens to prepare antibodies for use as diagnostic probes and even as molecular weight markers. Being able to specifically substitute one or more amino acids in the PTH polypeptide permits construction of specific molecular weight polypeptides.

In particular, the compounds of this invention are indicated for the prophylaxis and therapeutic treatment of osteoporosis and osteopenia in humans. Furthermore, the compounds of this invention are indicated for the prophylaxis and therapeutic treatment of other bone diseases. The compounds of this

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invention are also indicated for the prophylaxis and therapeutic treatment of hypoparathyroidism. Finally, the compounds of this invention are indicated for use as agonists for fracture repair and as antagonists for hypercalcemia.

In general, compounds of the present invention, or salts thereof, are administered in amounts between about 0.01 and 1 μg/kg body weight per day, preferably from about 0.07 to about 0.2 μg/kg body weight per day. For a 50 kg human female subject, the daily dose of biologically active compound is from about 0.5 to about 50 μgs, preferably from about 3.5 to about 10 μgs. In other mammals, such as horses, dogs, and cattle, higher doses may be required. This dosage may be delivered in a conventional pharmaceutical composition by a single administration, by multiple applications, or via controlled release, as needed to achieve the most effective results, preferably one or more times daily by injection. For example, this dosage may be delivered in a conventional pharmaceutical composition by nasal insufflation.

The selection of the exact dose and composition and the most appropriate delivery regimen will be influenced by, *inter alia*, the pharmacological properties of the selected compounds of the invention, the nature and severity of the condition being treated, and the physical condition and mental acuity of the recipient.

Representative preferred delivery regimens include, without limitation, oral, parenteral, subcutaneous, transcutaneous, intramuscular and intravenous, rectal, buccal (including sublingual), transdermal, and intranasal insufflation.

Pharmaceutically acceptable salts retain the desired biological activity of the compounds of the invention without toxic side effects. Examples of such salts are (a) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; and salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acids, naphthalene disulfonic acids, polygalacturonic acid and the like; (b) base addition salts formed with

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polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, and the like; or with an organic cation formed from N,N'-dibenzylethylenediamine or ethylenediamine; or (c) combinations of (a) and (b), e.g., a zinc tannate salt and the like. Pharmaceutically acceptable buffers include but are not limited to saline or phosphate buffered saline. Also included in these solutions may be acceptable preservative known to those of skill in the art.

A further aspect of the present invention relates to pharmaceutical compositions comprising as an active ingredient compounds of the invention or derivatives thereof of the present invention, or pharmaceutically acceptable salt thereof, in admixture with a pharmaceutically acceptable, non-toxic carrier. As mentioned above, such compositions may be prepared for parenteral (subcutaneous, transcutaneous, intramuscular or intravenous) administration, particularly in the form of liquid solutions or suspensions; for oral or buccal administration, particularly in the form of tablets or capsules; for rectal, transdermal administration; and for intranasal administration, particularly in the form of powders, nasal drops or aerosols.

The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well-known in the pharmaceutical art, for example as described in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., (1985), incorporated herein by reference. Formulations for parenteral administration may contain as excipients sterile water or saline, alkylene glycols such as propylene glycol, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. For oral administration, the formulation can be enhanced by the addition of bile salts or acylcarnitines. Formulations for nasal administration may be solid and may contain excipients, for example, lactose or dextran, or may be aqueous or oily solutions for use in the form of nasal drops or metered spray. For buccal administration typical excipients include sugars, calcium stearate, magnesium stearate, pregelatinated starch, and the like.

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When formulated for the most preferred route of administration, nasal administration, the absorption across the nasal mucous membrane may be enhanced by surfactant acids, such as for example, glycocholic acid, cholic acid, taurocholic acid, ethocholic acid, deoxycholic acid, chenodeoxycholic acid, dehydrocholic acid, glycodeoxycholic acid, cyclodextrins and the like in an amount in the range between about 0.2 and 15 weight percent, preferably between about 0.5 and 4 weight percent, most preferably about 2 weight percent.

Delivery of the compounds of the present invention to the subject over prolonged periods of time, for example, for periods of one week to one year, may be accomplished by a single administration of a controlled release system containing sufficient active ingredient for the desired release period. Various controlled release systems, such as monolithic or reservoir-type microcapsules, depot implants, osmotic pumps, vesicles, micelles, liposomes, transdermal patches, iontophoretic devices and alternative injectable dosage forms may be utilized for this purpose. Localization at the site to which delivery of the active ingredient is desired is an additional feature of some controlled release devices, which may prove beneficial in the treatment of certain disorders.

One form of controlled release formulation contains the polypeptide or its salt dispersed or encapsulated in a slowly degrading, non-toxic, non-antigenic polymer such as copoly(lactic/glycolic) acid, as described in the pioneering work of Kent, Lewis, Sanders, and Tice, U.S. Pat. No. 4,675,189. The compounds or, preferably, their relatively insoluble salts, may also be formulated in cholesterol or other lipid matrix pellets, or silastomer matrix implants. Additional slow release, depot implant or injectable formulations will be apparent to the skilled artisan. See, for example, Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson ed., Marcel Dekker, Inc., New York, 1978, and R. W. Baker, Controlled Release of Biologically Active Agents, John Wiley & Sons, New York, 1987.

Like PTH, the PTH variants may be administered in combination with other agents useful in treating a given clinical condition. When treating osteoporosis and other bone-related disorders for example, the PTH variants may

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be administered in conjunction with a dietary calcium supplement or with a vitamin D analog (*see* U.S. Pat. No. 4,698,328). Alternatively, the PTH variant may be administered, preferably using a cyclic therapeutic regimen, in combination with bisphosphonates, as described for example in U.S. Pat. No. 4,761,406, or in combination with one or more bone therapeutic agents such as, without limitation, calcitonin and estrogen.

*PTH Analog Receptor-Signaling Activities*

A crucial step in the expression of hormonal action is the interaction of hormones with receptors on the plasma membrane surface of target cells. The formation of hormone-receptor complexes allows the transduction of extracellular signals into the cell to elicit a variety of biological responses.

Polypeptides described herein can be screened for their agonistic or antagonistic properties using the cAMP accumulation assay. Cells expressing PTH-1 receptor on the cell surface are incubated with native PTH(1-84) for 5-60 minutes at 37°C., in the presence of 2 mM IBMX (3-isobutyl-1-methyl-xanthine, Sigma, St. Louis, MO). Cyclic AMP accumulation is measured by specific radio-immunoassay. A compound that competes with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor, and that inhibits the effect of native PTH(1-84) or PTH(1-34) on cAMP accumulation, is considered a competitive antagonist. Such a compound would be useful for treating hypercalcemia.

Conversely, a PTH analog described herein or a derivative thereof that does not compete with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor, but which still prevents native PTH(1-84) or PTH(1-34) activation of cAMP accumulation (presumably by blocking the receptor activation site) is considered a non-competitive antagonist. Such a compound would be useful for treating hypercalcemia.

The compounds described herein that compete with native PTH(1-84) or PTH(1-34)) for binding to the PTH-1 receptor, and which stimulates cAMP accumulation in the presence or absence of native PTH(1-84) or PTH(1-34) are

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competitive agonists. A compound that does not compete with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor but which is still capable of stimulating cAMP accumulation in the presence or absence of native PTH(1-84) or PTH(1-34), or which stimulates a higher cAMP accumulation than that observed by a compound of the invention or a derivative thereof alone, would be considered a non-competitive agonist.


### *Therapeutic Uses of PTH Analogs*


Some forms of hypercalcemia and hypocalcemia are related to the interaction between PTH and PTHrP and the PTH-1 and receptors. Hypercalcemia is a condition in which there is an abnormal elevation in serum calcium level; it is often associated with other diseases, including hyperparathyroidism, osteoporosis, carcinomas of the breast, lung and prostate, epidermoid cancers of the head and neck and of the esophagus, multiple myeloma, and hypernephroma. Hypocalcemia, a condition in which the serum calcium level is abnormally low, may result from a deficiency of effective PTH, *e.g.*, following thyroid surgery.

By "agonist" is intended a ligand capable of enhancing or potentiating a cellular response mediated by the PTH-1 receptor. By "antagonist" is intended a ligand capable of inhibiting a cellular response mediated by the PTH-1 receptor. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit such a cellular response can be determined using art-known protein ligand/receptor cellular response or binding assays, including those described elsewhere in this application.

In accordance with yet a further aspect of the invention, there is provided a method for treating a medical disorder that results from altered or excessive action of the PTH-1 receptor, comprising administering to a patient therapeutically effective amount of a compound of the invention or a derivative thereof sufficient to inhibit activation of the PTH-1 receptor of said patient.

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In this embodiment, a patient who is suspected of having a disorder resulting from altered action of the PTH-1 receptor can be treated using compounds of the invention or derivatives thereof of the invention which are a selective antagonists of the PTH-1 receptor. Such antagonists include compounds of the invention or derivatives thereof of the invention which have been determined (by the assays described herein) to interfere with PTH-1 receptor-mediated cell activation or other derivatives having similar properties.

To administer the antagonist, the appropriate compound of the invention or a derivative thereof is used in the manufacture of a medicament, generally by being formulated in an appropriate carrier or excipient such as, *e.g.*, physiological saline, and preferably administered intravenously, intramuscularly, subcutaneously, orally, or intranasally, at a dosage that provides adequate inhibition of a compound of the invention or a derivative thereof binding to the PTH-1 receptor. Typical dosage would be 1 ng to 10 mg of the peptide per kg body weight per day.

In accordance with yet a further aspect of the invention, there is provided a method for treating osteoporosis, comprising administering to a patient a therapeutically effective amount of a compound of the invention or a derivative thereof, sufficient to activate the PTH-1 receptor of said patient. Similar dosages and administration as described above for the PTH/PTHrP antagonist, can be used for administration of a PTH/PTHrP agonist, *e.g.*, for treatment of conditions such as osteoporosis, other metabolic bone disorders, and hypoparathyroidism and related disorders.

It will be appreciated to those skilled in the art that the invention can be performed within a wide range of equivalent parameters of composition, concentration, modes of administration, and conditions without departing from the spirit or scope of the invention or any embodiment thereof.

Having now fully described the invention, the same will be more readily understood by reference to specific examples which are provided by way of illustration, and are not intended to be limiting of the invention, unless herein specified.

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## *Examples*

The following protocols and experimental details are referenced in the examples that follow.

Peptides. Each peptide utilized in this study contained a free amino acid
5    terminus and a carboxamide at the C-terminus. Peptides were prepared on automated peptide synthesizers (model 430A PE, Applied Biosystems, Foster City, CA, or Model 396 MBS Advanced Chem Tect, Louisville, KY) using Fmoc main-chain protecting group chemistry, HBTU/HOBt/DIEA (1:1:2 molar ratio) for coupling reactions, and TFA-mediated cleavage/sidechain-deprotection (MGH
10   Biopolymer Synthesis Facility, Boston, MA). All peptides were desalted by adsorption on a C18-containing cartridge, and purified further by HPLC. The dry peptide powders were reconstituted in 10 mM acetic acid and stored at $-80\,^\circ$C. The purity, identity, and stock concentration for each peptide was secured by analytical HPLC, Matrix-assisted laser desorption/ionization (MALDI) mass
15   spectrometry and amino acid analysis. Radiolabeling of [M]PTH(1-21) and [Aib$^{1,3}$,M]PTH(1-21) was performed using $^{125}$I-Na (2,200 Ci/mmol, NEN) and chloramine-T; the resultant radioligands were purified by HPLC.

Cell Culture. The cell line HKRK-B28 (Takasu, H., *et al., J. Bone Miner. Res. 14*:11-20 (1999)) was derived from the porcine kidney cell line, LLC-
20   PK$_1$ by stable transfection with plasmid DNA encoding the human P1R and expresses ~280,000 receptors per cell. These cells, as well as COS-7 cells and SaOS-2-B10 cells, were cultured at $27\,^\circ$C in T-75 flasks (75 mm$^2$) in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%), penicillin G (20 units/ml), streptomycin sulfate (20 $\mu$g/ml) and amphotericin B
25   (0.05 $\mu$g/ml) in a humidified atmosphere containing 5% $CO_2$. Stock solutions of EGTA/trypsin and antibiotics were from GIBCO; fetal bovine serum was from Hyclone Laboratories (Logan, UT). COS-7 cells sub-cultured in 24-well plates were transfected with plasmid DNA (200 ng per well) encoding the wild-type human P1R or truncated human P1R deleted for residues (24-181) (Shimizu, M.,
30   *et al., J. Biol. Chem. 275*:21836-21843 (2000)) that was purified by cesium

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chloride/ethidium bromide density gradient centrifugation, and FuGENE 6 transfection reagent (Roche Indianapolis IN) according to the manufacturer's recommended procedure. All cells, in 24-well plates, were treated with fresh media and shifted to 33 °C for 12 to 24 h prior to assay.

cAMP Stimulation. Stimulation of cells with peptide analogs was performed in 24-well plates. Cells were rinsed with 0.5 mL of binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, 0.5% fetal bovine serum, adjusted to pH 7.5 with HCl) and treated with 200 µL of cAMP assay buffer (Delbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, 1 mg/mL bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) and 100 µL of binding buffer containing varying amounts of peptide analog (final volume = 300µL). The medium was removed after incubation for 30 to 60 minutes at room temperature, and the cells were frozen on dry ice, lysed with 0.5 mL 50 mM HCl, and refrozen (~80°C). The cAMP content of the diluted lysate was determined by radioimmunoassay. The $EC_{50}$ response values were calculated using nonlinear regression (see below).

Competition Binding. Binding reactions were performed with HKRK-B28 cells or in COS-7 cells in 24-well plates. The cells were rinsed with 0.5 mL of binding buffer, and then treated successively with 100 µL binding buffer, 100 µL of binding buffer containing various amounts of unlabeled competitor ligand, and 100 µL of binding buffer containing ca. 100,000 cpm of $^{125}I$-[M]PTH(1-21) or $^{125}I$-[Aib$^{1,3}$,M]PTH(1-21)} (ca. 26 fmol; final volume = 300 µL). Incubations were 4 to 6 h at 4°C, at which time near equilibrium conditions were attained. Cells were then placed on ice, the binding medium was removed, and the monolayer was rinsed three times with 0.5 mL of cold binding buffer. The cells were subsequently lysed with 0.5 mL 5N NaOH and counted for radioactivity. For each tracer and in each experiment, the non-specific binding was determined as the radioactivity that bound in the presence of the same unlabeled peptide at a concentration of 1 µM, and was ~1% of total radioactivity added for each tracer. The maximum specific binding ($B_0$) was the total radioactivity bound in the absence of competing ligand, corrected for nonspecific binding, and for each

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tracer, ranged from 8% to 20% of the total radioactivity added. Nonlinear regression was used to calculate binding $IC_{50}$ values (see below). Scatchard transformations of homologous competition binding data derived from studies with 26 fmol of $^{125}I$-[Aib$^{1,3}$,M]PTH(1-21) were employed for estimations of apparent equilibrium dissociation constant ($k_{Dapp}s$) and total number of ligand binding sites ($B_{max}$), assuming a single class of binding sites and equal affinities of the iodinated and non iodinated ligand.

Stimulation of Inositol Phosphate Production. COS-7 cells transfected as above with P1R-WT were treated with serum-free, inositol-free DMEM containing 0.1% bovine serum albumin and [$^3$H]myo-inositol (NEN, Boston, MA) (2 $\mu$Ci/mL) for 16 h prior to assay. At the time of the assay, the cells were rinsed with binding buffer containing LiCl (30 mM) and treated with the same buffer with or without a PTH analog. The cells were then incubated at 37°C for 40 min, after which the buffer was removed and replaced by 0.5 mL of ice cold 5% trichloroacetic acid solution. After 3 h on ice, the lysate was collected and extracted twice with ethyl ether. The lysate was then applied to an ion exchange column (0.5 mL resin bed) and the total inositol phosphates were eluted as described previously (Berridge, M.J., *et al., Biochem. J. 212*:473-482 (1983)), and counted in liquid scintillation cocktail.

Inhibition of Chondrocyte Differentiation in Embryonic Mouse Metatarsals. Metatarsals from embryonic day (E) 15.5 mouse embryos were excised and cultured in a 37°C humidified incubator (5% $CO_2$) in serum-free $\alpha$MEM media in 24 well plates. Sixteen hours later, a PTH analog or vehicle was added, and the samples were incubated for an additional 48 h in 37°C with peptide or vehicle added again at the 24 h time point. At the end of the 64 h incubation period, the samples were fixed with 10% formalin/phosphate-buffered saline, then directly visualized on a dissecting microscope using white light. Sections were processed for in-situ hybridization analysis using $^{35}$S-labeled riboprobes specific for collagen type X mRNA, a developmental marker gene expressed only in hypertrophic chrondrocytes of the growth plate.

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Circular Dichroism. Circular Dichroism spectra were recorded on a Jasco model 710 spectropolarimeter; peptides were analyzed at a concentrtion of 20 μM in 50 mM sodium phosphate buffer pH 7.4, or the same buffer containing 2,2,2-trifluoroethanol at 20% (v/v). Spectroscopic scans were performed at 20°C and

5 at wavelengths between 185 and 255 nM, with data recored at each 1 nM interval. The spectral bandwidth was 1.5 nM and 8 scans were accumulated and averaged for each sample. At each wavelength, the mean residue elipticity [θ x 100/$l$ x $C$ x n); where θ is the raw elipticity value (in dimensions of millidegree), $l$ is the sample path length, $C$ = is the molar peptide concentration, and n is the number

10 of residues in the peptide (Bowen, W.P., and Jerman, J.C., *Trends in Pharmacol. Sci. 16*: 413-417 (1995)). The helical content of each peptide was estimated by dividing [θ] observed at 222 nM for that peptide by -28,100, which is the reported [θ]$_{222}$obs for a model helical decapeptide (Bowen, W.P., and Jerman, J.C., *Trends in Pharmacol. Sci. 16*: 413-417 (1995)).

15 Data Calculation. Calculations were performed using Microsoft® Excel. Nonlinear regression analyses of binding and cAMP dose-response data were performed using the four-parameter equation: $y_p$ = Min + [(Max - Min)/(1 + (IC$_{50}$/$x$)$^{slope}$)]. The Excel Solver function was utilized for parameter optimization, as described previously (Carter, P.H., *et al., Endocrinology 140*: 4972-4981

20 (1999); Bowen, W.P., and Jerman, J.C., *Trends in Pharmacol. Sci. 16*: 413-417 (1995)). Differences between paired data sets were statistically evaluated using a one-tailed Student's t-test, assuming unequal variances for the two sets.


### Example 1: Aib-scan in [M]PTH(1-14)


The effect of introducing individual Aib substitutions at each position in

25 the scaffold peptide [M]PTH(1-14) (Shimizu, M., *et al., Endocrinology* (2001) (In Press))) were analyzed. In cAMP stimulation assays in HKRK-B28 cells, the parent peptide [M]PTH(1-14) stimulated approximately the same (~70-fold) maximum (Emax) increase in intracellular cAMP that was induced by PTH(1-34), but the potency (EC$_{50}$) of the shorter peptide was 40-fold less than that of

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PTH(1-34) ($EC_{50}$ s= 100±20 and 2.5±0.4 nM, respectively) (Fig 1 and Table 1). Most of the Aib substitutions diminished potency. Severe reductions occured with Aib substitutions at positions 6, 8 and 9 (all > 2,300-fold), moderate reductions occurred with substitution at positions 2, 4, 5 and 11 (all 170 to 670-fold) and minor reductions occurred with substitutions at positions 7, 10, 12, 13 and 14 (all <3-fold; Table 1). Substitution of Aib at positions 1 and 3 resulted in peptides with 10- and 8-fold improvements in potency, relative to [M]PTH(1-14), respectively ($P \le 0.01$). These Aib-scan data extend previous alanine-scan and substitution analyses of PTH(1-14) analogs, in which residues in the (1-9) region, excluding residue 3, were found to be intolerant to substitution, and residues 3 and 10-14 were found to be relatively tolerant (Luck, M.D., *et al., Molecular Endocrinology 13*:670-680 (1999); Shimizu, M., *et al., J. Biol. Chem. 275*:19456-19460 (2000); Pellegrini, M., *et al., J. Biol. Chem. 273*:10420-10427 (1998)).

The P1R-binding properties of these analogs were assayed in competition studies performed in HKRK-B8 cells. In previous studies, PTH(1-14) binding could not be detected using $^{125}$I-PTH(1-34) and related N-terminally intact and relatively unmodified radioligands (Luck, M.D., *et al., Molecular Endocrinology 13*:670-680 (1999)). However, measurable PTH(1-14) binding was observed with $^{125}$I-PTH(3-34) used as a tracer radioligand (Hoare, S.R.J., *et al., J. Biol. Chem 276*:7741-7753 (2001); Shimizu, M., *et al., Endocrinology* (2001) (In Press)). Receptor binding affinity was assessed using a tracer radioligand that was structurally more homologous to the [M]PTH(1-14) analogs being investigated. The radiolabeled peptide $^{125}$I-[Ala$^{3,12}$Nle$^8$Gln$^{10}$,Har$^{11}$,Trp$^{14}$,Tyr$^{15}$]PTH(1-15)amide was evaluated, but did not bind detectably to HKRK-B28 cells. A similar analog, which was extended to position 21 and contained the affinity-enhancing substitution of Glu$^{19}$→Arg (Takasu, H., *et al., Biochemistry 38*:13453-13460 (1999); Kronenberg, H.M., *et al., Recent Prog. Horm. Res. 53*:283-301 (1998)), was prepared. The resulting radioligand $^{125}$I-[M]PTH(1-21) bound adequately to the P1R expressed intact HKRK-B28 cells, as the amount of specifically bound radioactivity (e.g. that which could be inhibited by excess unlabeled [M]PTH(1-21) peptide), was ~15%

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to 20% of total radioactivity added, and that which bound to untransfected LLC-PK1 cells was <2% of total added. Thus, this tracer ligand was suitable for competition analyses.

The binding of $^{125}$I-[M]PTH(1-21) to HKRK-B28 cells was fully inhibited
5    by PTH(1-34) ($IC_{50}$ = 18±3 nM) and more weakly but to near completion by
[M]PTH(1-14) ($IC_{50}$ = 13,000±3,000 nM, Table 1). Relative to the apparent binding affinity of [M]PTH(1-14), most of the Aib substitutions reduced affinity, in accordance with the corresponding effects on cAMP-signaling potency (Table 1). The only Aib substitutions that improved affinity significantly were
10   those at positions 1 and 3 (13- and 8-fold, respectively, Aib at position 10 showed a trend towards causing a 1.4-fold improvement in affinity, $P$ = 0.16). Strong (>10-fold) reductions in affinity occurred with Aib substitutions at positions 4, 7, 8 and 9, while mild (<10-fold) reductions occurred with the Aib at positions 2, 5, 12, 13 and 14. While most of the Aib substitutions had effects on receptor-
15   binding affinity that were approximately proportional to their effects on cAMP-stimulating potency, those at positions 2 and 6 had less of an effect on binding than on potency. Thus, these two substitutions reduced affinity ~3-fold, relative to [M]PTH(1-14), while they reduced potency ~470- and ~2,300-fold, respectively (Table 1).

20       Combining the Aib substitutions at positions 1 and 3 revealed an additive effects, as [Aib$^{1,3}$,M]PTH(1-14) was 90-fold more potent in stimulating cAMP formation than was [M]PTH(1-14) ($EC_{50}$ s = 1.1±0.1 nM, 100±20 nM, respectively), and at least as potent as PTH(1-34) ($EC_{50}$ = 2.5±0.4 nM, $P$ = 0.01, Fig. 2A and Tale 1). The effects of the single Aib substitutions at position 1 and
25   3 on receptor-binding affinity were also additive, as [Aib$^{1,3}$,M]PTH(1-14) bound with 100-fold higher apparent affinity than did [M]PTH(1-14) (Fig. 2B and Table 1). Aib substitutions were subsequently introduced at positions 1 and 3 in [M]PTH(1-11) analog to determine if the paired substitution could enhance activity of the shorter peptide sequence. Previously, it was shown that while
30   native PTH peptides shorter than (1-14) were devoid of cAMP-stimulating activity (Luck, M.D., *et al.*, *Molecular Endocrinology* *13*:670-680 (1999)),

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modified PTH(1-11) analogs, such as [M]PTH(1-11) could induce a full cAMP response in HKRK-B28 cells, albeit with a potency ($EC_{50}$ = 3 µM) nearly 1,000-fold weaker than that of PTH(1-34)(Shimizu, M., *et al., Endocrinology* (2001) (In Press)). In cAMP stimulation assays in HKRK-B28 cells, [Aib[1,3],M]PTH(1-11) was fully efficacious and its potency ($EC_{50}$ 4.0±0.8 nM) was 1,000-fold greater than that of [M]PTH(1-11) (Shimizu, M., *et al., Endocrinology* (2001) (In Press)) and nearly equal to that of PTH(1-34) (Fig. 2A, Table 1). The Aib-1,3 modification also enhanced potency of PTH(1-10) analog, as [Aib[1,3],Gln[10]]PTH(1-10) was 50-fold more potent than our previously most potent PTH(1-10) analog, [Ala[3],Gln[10]]PTH(1-10) ($EC_{50}$ s=16±2 µM and ~ 800 µM, respectively) (Shimizu, M., *et al., Endocrinology* (2001) (In Press)) (Fig. 2A, Table 1). The 4000-fold weaker potency that [Aib[1,3],Gln[10]]PTH(1-10) exhibited, relative to that of [Aib[1,3],M]PTH(1-11), indicated the importance of the position 11 residue (homoarginine) in the activities of the Aib-containing peptides. Little or no stimulation of cAMP accumulation was observed with [Aib[1,3]]PTH(1-9) (Fig. 2A and Table 1). In competition binding assays, [Aib[1,3],M]PTH(1-11) effectively inhibited [125]I-[Aib[1,3],M[PTH(1-21) binding to HKRK-B8 cells ($IC_{50}$ = 970±300 nM), but [Aib[1,3],Gln[10]]PTH(1-10) and [Aib[1,3]]PTH(1-9) did not bind detachably (Fig. 2B and Table 1).

Table 1 cAMP Stimulation and hP1R-Binding Properties in HKRK-B28 cells

| | cAMP | | | Binding | |
|---|---|---|---|---|---|
| Peptide | $EC_{50}$ nM | $EMax_{(obs.,)}$ pmole/well | | $IC_{50}$ nM | |
| # | | | (n) | | (n) |
| 92    PTH(1-34) | 2.5 ± 0.4 | 280 ± 11 | 10 | 18 ± 3 | 7 |
| 621   [M]PTH(1-14) | 100 ± 20 | 270 ± 8 | 10 | 13,000 ± 3,000 | 4 |
| *Aib scan in [M]PTH(1-14)* | | | | | |
| 622   Aib-1 | 10 ± 3 | 273 ± 6 | 6 | 980 ± 160 | 3 |
| 623   Aib-2 | 47,000 ± 13,000 | 168 ± 6 | 3 | 50,000 ± 11,000 | 3 |
| 624   Aib-3 | 13 ± 3 | 269 ± 7 | 6 | 1,700 ± 200 | 3 |
| 625   Aib-4 | 17,000 ± 3,400 | 221 ± 10 | 3 | 148,000 ± 40,000 | 3 |
| 626   Aib-5 | 66,000 ± 38,000 | 169 ± 18 | 3 | N.B. | 3 |
| 627   Aib-6 | 230,000 ± 78,000 | 116 ± 12 | 3 | 31,000 ± 7,000 | 3 |
| 628   Aib-7 | 2,600 ± 980 | 275 ± 8 | 3 | 490,000 ± 170,000 | 3 |
| 629   Aib-8 | 1,500,000 ± 970,000 | 34 ± 7 | 3 | N.B. | 3 |
| 630   Aib-9 | 710,000 ± 330,000 | 51 ± 8 | 4 | N.B. | 3 |
| 631   Aib-10 | 3,000 ± 2,100 | 214 ± 22 | 3 | 9,100 ± 1,500 | 3 |
| 632   Aib-11 | 67,000 ± 51,000 | 96 ± 18 | 4 | N.B. | 3 |
| 633   Aib-12 | 440 ± 300 | 263 ± 9 | 3 | 15,000 ± 3,000 | 3 |
| 634   Aib-13 | 480 ± 250 | 259 ± 10 | 4 | 44,000 ± 8,000 | 3 |
| 635   Aib-14 | 350 ± 100 | 273 ± 6 | 3 | 79,000 ± 27,000 | 3 |
| *Aib-1,3 in [M]PTH(1-X)* | | | | | |
| 608   [M]PTH(1-21) | N.D. | | | 53 ± 4 | 3 |
| 674   [Aib[1,3], M]PTH (1-21) | 4.3 ± 1.6 | 284 ± 76 | 3 | 31 ± 7 | 3 |
| 671   [Aib[1,3], M]PTH (1-14) | 1.1 ± 0.1 | 278 ± 20 | 5 | 130 ± 20 | 5 |
| 682   [Aib[1,3], M]PTH (1-11) | 4.0 ± 0.8 | 243 ± 15 | 4 | 970 ± 300 | 3 |
| 684   [Aib[1,3], M]PTH (1-10) | 16,000 ± 2,000 | 111 ± 8 | 4 | N.B. | 3 |
| 696   [Aib[1,3]]PTH (1-9) | >10,000 | 10 ± 1 | 3 | N.B. | 3 |

Peptides PTH(1-34) ([Nle[8,21], Tyr[34]]PTH(1-34)amide), [M]PTH(1-14) (M = Ala[3,12], Gln[10], Har[11], Trp[14]), and [M]PTH(1-14 analogs, or C-terminally truncated derivatives thereof, containing α-aminoisobutyric acid (Aib) substitutions at the indicated positions were functionally evaluated in HKRK-B28 cells. "M" in [Aib[1,3],M]PTH(1-21) = Nle[8], Gln[10], Har[11], Ala[12], Trp[14], Arg[19] and Tyr[21]. The basal cAMP values (not subtracted) were 4.0 ± 0.1 pmole/well (n=10). Peptides were based on the rat PTH sequence and were carboxy-amidated. Competition binding analyses were performed with [125]I-[M]PTH(1-21)amide (Ala[1,3]) as tracer for 4h at 4°C. Data are means (±S.E.M.) Of the number of experiments indiated (n). N.B., no binding was detected at a peptide concentration of 10 μm; N.D.; the experiment was not done. The sequence of MPTH(1-14) is: Ala-Val-Ala*-Glu-Ile-Gln-Leu-Met-His-Gln*-Har*-Ala*-Lys-Trp* where the asterisk denotes substituted amino acids are not found at that position in native rat PTH(1-14).

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## Example 2: Analog Activity in COS-7 Cells

The possibility that the activity-enhancing effects of the Aib substitutions at positions 1 and 3 were mediated through the juxtamembrane (J) region of the receptor was investigated in COS-7 cells transiently transfected with P1R-delNt. P1R-delNt was a truncated P1R that lacked most of the amino-terminal extracellular domain. With this receptor construct, PTH(1-34) was a much weaker agonist than it was with P1R-WT, while other PTH(1-14) analogs exhibited approximately the same potency with P1R-delNt as with P1R-WT (Kaul, R., and Balaram, P., *Bioorganic & Medicinal Chemistry* 7:105-117 (1999)). Consistent with this, the cAMP-stimulating potency of [Aib$^{1,3}$,M]PTH(1-14) on P1R-delNt ($EC_{50}$=0.73±0.16 nM) was comparable to its potency on COS-7 cells expressing P1R-WT (1.2±0.6 nM) (Table 2). On P1R-delNt, [Aib$^{1,3}$,M]PTH(1-14) was 55-fold more potent than was [M]PTH(1-14) ($EC_{50}$=40±2 nM, Fig. 3A and Table 2). This result indicated that the potency-enhancing effects of the Aib-1,3 substitutions were exerted through the J domain of the receptor. Remarkably, [Aib$^{1,3}$,M]PTH(1-14) was as potent on P1R-delNt as PTH(1-34) was on P1R-WT ($EC_{50}$s=0.73±0.16 nM and 1.4±0.7 nM, respectively, $P$=0.4, Table 2) and the EMax induced by [Aib$^{1,3}$,M]PTH(1-14) on P1R-delNt was equal to that induced by PTH(1-34) on P1R-WT (250±20 picomole/well and 240±50 picomole/well, respectively, $P$=0.7, Table 2). As expected, PTH(1-34), was ~500-fold weaker on P1R-delNt than P1R-WT ($EC_{50}$s=680±110 nM and 1.4±0.7 nM, respectively; Fig. 3A and Table 2).

Heretofore, it was not possible to demonstrate a PLC response for any PTH analog in cells expressing P1R-delNt, including [M]PTH(1-14). The analog [Aib$^{1,3}$,M]PTH(1-14), however, induced an approximate 3-fold increase in inositol phosphate (IP) production, relative to the basal level of IPs, in COS-7 cells expressing P1R-delNt, while, as expected, PTH(1-34) and [M]PTH(1-14) were inactive (Fig. 3B). Thus, the truncated receptor can couple to the PCL signaling pathway when stimulated with the Aib-containing PTH peptide. With P1R-WT, both [Aib$^{1,3}$,M]PTH(1-14) and [M]PTH(1-14) stimulated the same 4-

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fold increase in IP formation that was observed for PTH(1-34) acting on this receptor, and with this receptor, [Aib$^{1,3}$,M]PTH(1-14) was 66-fold more potent than [M]PTH(1-14) ($EC_{50}$=71±9 nM, and 4,700±2,000 nM, respectively, Table 2). Thus, the Aib-1,3 substitutions enhance the ligand's capacity to

5      stimulate PLC activity with P1R-WT, as well as with P1R-delNt.

The radioligand used in the above binding studies in HKRK-B8 cells, $^{125}$I-[M]PTH(1-21), did not bind detectably to P1R-delNt. To potentially improve affinity of this peptide, the paired Aib-1,3 modifications were introduced to produce [Aib$^{1,3}$,M]PTH(1-21). The corresponding radioiodinated peptide, $^{125}$I-

10     [Aib$^{1,3}$,M]PTH(1-21), bound to COS-7 cells expressing P1R-WT; thus, the specific binding of this tracer (e.g. that which could be inhibited by excess unlabeled [Aib$^{1,3}$,M]PTH(1-21) peptide) was ~10% and ~15% of the total radioactivity added, for each receptor, respectively. The total specific binding observed in COS-7 cells transfected with vector DNA alone was <2% of total

15     radioactivity added. This radioligand, therefore, enabled competition binding experiments to be performed with both the wild-type and truncated PTH-1 receptors. Scatchard transformation of homologous competition binding data obtained with $^{125}$I-[Aib$^{1,3}$,M]PTH(1-21) as tracer radioligand and varying amounts of unlabeled [Aib$^{1,3}$,M]PTH(1-21) indicated that the ligand's affinity at P1R-delNt

20     was slightly (<2-fold) weaker than it was at P1R-WT ($K_{Dapp}$s=29±3 and 17±2 nM, respectively, $P$=0.01), while the corresponding $B_{max}$ values for the two receptors were not significantly different (1.3±0.1x10$^6$ receptors/cell and 1.9±0.8x10$^6$ receptors/cell respectively, $P$=0.3).

At the truncated receptor, [Aib$^{1,3}$,M]PTH(1-14) and [M]PTH(1-14), in

25     addition to [Aib$^{1,3}$,M]PTH(1-21), effectively inhibited the binding of $^{125}$I-[Aib$^{1,3}$,M]PTH(1-21), whereas, PTH(1-34) did not (Fig. 3C). Like [Aib$^{1,3}$,M]PTH(1-21), the apparent binding affinities that [Aib$^{1,3}$,M]PTH(1-14) and [M]PTH(1-14) exhibited at P1R-delNt were comparable to the corresponding affinities observed at the wild-type P1R (Table 2). At both P1R-delNt and P1R-

30     WT, the binding affinities of [Aib$^{1,3}$,M]PTH(1-14) were ~10-fold stronger than

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the corresponding affinities observed for [M]PTH(1-14). The Aib substitutions therefore enhanced the ligand's binding affinity for the J domain of the P1R.


Table 2 Functional properties of PTH analogs in COS-7 cells

| Peptide[a] | cAMP | | | PLC | | | Binding | |
|---|---|---|---|---|---|---|---|---|
| | $EC_{50}$ | $EMAX_{(obs.)}$ | | $EC_{50}$ | $EMAX_{(obs.)}$ | | $IC_{50}$ | |
| | *nM* | *pmole/well* | *(n)* | *nM* | *foldxbasal* | *(n)* | *nM* | *(n)* |
| *hP1R-WT* | | | | | | | | |
| 92.5  PTH(1-34) | 1.4 ± 0.7 | 240 ± 50 | 3 | 17 ± 2 | 4.2 ± 0.6 | 3 | 12 ± 3 | 4 |
| 521  PTH(1-14) | 90,000 ± 34,000 | 140 ± 30 | * 3 | --- | | | N.B. | 3 |
| 621  [M]PTH(1-14) | 49 ± 21 | 240 ± 50 | 3 | 4,700 ± 2,000 | 3.9 ± 0.5 | 3 | 20,000 ± 3,000 | 3 |
| 674  [Aib$^{1,3}$M]PTH(1-21) | 0.8 ± 0.1 | 300 ± 30 | 3 | N.D. | | | 28 ± 5 | 6 |
| 671  [Aib$^{1,3}$M]PTH(1-14) | 1.2 ± 0.6 | 240 ± 40 | 3 | 71 ± 9 | 4.3 ± 0.6 | 3 | 2,250 ± 1,100 | 4 |
| 682  [Aib$^{1,3}$M]PTH(1-11) | 2.1 ± 0.7 | 190 ± 40 | 3 | N.D. | | | 16,000 ± 1,000 | 3 |
| 684  [Aib$^{1,3}$M]PTH(1-10) | 100,000 ± 40,000 | 120 ± 10 | * 3 | N.D. | | | N.B. | 3 |
| *hP1R-delNT* | | | | | | | | |
| 92.5  PTH(1-34) | 680 ± 110 | 220 ± 30 | 3 | --- | | 3 | N.B. | 4 |
| 521  PTH(1-14) | 140,000 ± 30,000 | 110 ± 10 | * 3 | --- | | 3 | N.B. | 3 |
| 621  [M]PTH(1-14) | 40 ± 2.0 | 220 ± 20 | 3 | --- | | 3 | 17,400 ± 1,400 | 3 |
| 674  [Aib$^{1,3}$M]PTH(1-21) | 0.38 ± 0.10 | 240 ± 20 | 3 | N.D. | | | 27 ± 1 | 6 |
| 671  [Aib$^{1,3}$M]PTH(1-14) | 0.73 ± 0.16 | 250 ± 20 | 3 | 130 ± 30 | 3.1 ± 0.2 | 3 | 1,600 ± 200 | 4 |
| 682  [Aib$^{1,3}$M]PTH(1-11) | 2.00 ± 0.40 | 220 ± 20 | 3 | N.D. | | | 13,000 ± 1,000 | 3 |
| 684  [Aib$^{1,3}$M]PTH(1-10) | 53,000 ± 10,000 | 84 ± 4 | * 3 | N.D. | | | N.B. | 3 |

The peptides were derivatives of rat PTH with C-terminal carboxamides; in PTH(1-14) and shorter analogs, "M" refers to the amino acid modifications: $Ala^{3,12}$,$Gln^{10}$,$Har^{11}$,$Trp^{14}$, unless the residue position was absent due to truncation or replaced by Aib (α-aminoisobutyric acid); in the PTH(1-21) analog, "M" refers to the same modifications and the modifications of $Nle^{8}$,$Arg^{19}$ and $Tyr^{21}$. Peptides were evaluated in COS-7 cells

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transiently transected with either the wild-type hP1R (hP1R-WT), or a truncated hP1R lacking most of the amino-terminal extracellular domain (hP1R-delNt).The basal levels of cAMP were $10.3 \pm 1.1$ and $9.5 \pm 1.3$ picomole per well for hP1R-WT and hP1R-delNt, respectively. The basal levels of $^3H$-inositol phosphates were $1,103 \pm 143$ and $2,929 \pm 877$ cpm per well for hP1R-WT and hP1R-delNt, respectively. The $Emax^{(obs.)}$ (maximum response observed) values in the cAMP and PLC assays were determined at ligand doses of 0.1 to 100 μM; an asterisk indicated that a plateau in the response curve was not attained and the curve-fitting equation used to determine the $EC_{50}$ was constrained to within one standard deviation of the maximum response observed with the same receptor in the same assay. Competition binding assays were performed with $^{125}I$-[$Aib^{1,3}$,M]PTH(1-21) radioligand as tracer. Values are means ($\pm$S.E.M.) of data from the number of independent experiments indicated ($n$), each of which was performed in duplicate. A dashed line indicates that no cAMP or PLC response was observed. N.B. indicates that no inhibition of tracer binding was observed. N.D. indicates that the experiment was not done.

## *Example 3: Activity in Bone Cells*

The number of PTH-1 receptors expressed on the surface of the PTH target cells in bone or kidney is uncertain, but it is likely to be considerably lower than that found in HKRK-B28 cells. Therefore, several of the Aib-mounted PTH analogs were evaluated using SaOS-2 cells. These cells were derived from a human osteosarcoma, exhibited osteoblast-like properties and endogenously expressed relatively low levels of the PTH-1 receptor (~20,000 receptors/cell (Marx, U.C., *et al., J. Biol. Chem. 273*:4308-4316 (1998)). In these cells, [$Aib^1$,M]PTH(1-14) and [$Aib^3$,M]PTH(1-14) were 15- and 8-fold more potent in stimulating cAMP formation than was [M]PTH(1-14), and [$Aib^{1,3}$,M]PTH(1-14) was 130-fold more potent than [M]PTH(1-14) (Fig. 4 and Table 3). Thus, in SaOS-2 cells, [$Aib^{1,3}$,M]PTH(1-14) was only 13-fold less potent than PTH(1-34) and at least five-orders of magnitude more potent than native PTH(1-14), for which no activity could be detected, even at a dose of 10 μM (Fig. 4).

Whether or not [$Aib^{1,3}$,M]PTH(1-14) activity could be detected in a more intact bone system was studied in an explant assay. An explant assay utilized cartilaginous metatarsal rudiments isolated from E15.5mouse embryos and subsequently cultured in multi-well plates containing serum-free media. A PTH peptide analog or vehicle control, was added to the culture 16 h after explantion, then again at 24 h. The incubation was terminated 24 h later for a total of 48 h of treatment over a 64 h period. In the absence of PTH, chondrocyte differentation occurred, such that by the end of the experiment, dense mineralization was apparent at the bone's mid-section (Fig. 5A). Differentiation

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was inhibited by the presence of PTH(1-34) (0.1 μM) or [Aib$^{1,3}$,M]PTH(1-14) (1

μM), as no mineralization was observed (Fig. 5, B and C). Mineralization was

also inhibited in these assays by [Aib$^{1,3}$,M]PTH(1-14), whereas no effect could

be detected for native PTH(1-14) (2 μM) (Fig. 5D). Comparable results were

obtained in each of three replicate experiments. In addition, mRNA *in situ*

hybridization analysis performed on the explanted metatarsals demonstrated that

both PTH(1-34) and [Aib$^{1,3}$,M]PTH(1-14) inhibited expression of the collagen X

gene, a bone developmental marker gene (data not shown). These inhibitory

effects were consistent with the known capacity of PTHrP to retard chondrocyte

differentiation in the growth plate cartilage of developing long bones (Pellegrini,

M., *et al., Biochemistry 37*:12737-12743 (1998)).

Table 3   cAMP Stimulation in SaOS-2 cells

|     |                                | $EC_{50}$    | $EMAX_{(obs.)}$ |     |
| --- | ------------------------------ | ------------ | --------------- | --- |
|     | **Peptide**                    | *nM*         | *pmole/well*    | **n** |
| 93  | **PTH (1-34)**                 | 0.2 ± 0.02   | 350 ± 30        | 4   |
| 621 | **[M]PTH(1-14)**               | 340 ± 120    | 340 ± 30        | 4   |
| 521 | **PTH(1-14)**                  | N.R.         |                 | 2   |
|     |                                |              |                 |     |
| 622 | **[Aib$^{1}$,M]PTH(1-14)**     | 22 ± 4       | 340 ± 30        | 4   |
| 624 | **[Aib$^{3}$,M]PTH(1-14)**     | 42 ± 8       | 330 ± 30        | 4   |
|     |                                |              |                 |     |
| 671 | **[Aib$^{1,3}$,M]PTH(1-14)**   | 2.6 ± 0.5    | 320 ± 30        | 3   |

The peptides PTH(1-34) ([Nle$^{8,21}$, Tyr$^{34}$]PTH(1-34)amide), [M]PTH(1-14) (m = Ala$^{3,12}$, Gln$^{10}$, Har$^{11}$, Trp$^{14}$), native PTH(1-14), and analogs of [M]PTH(1-14) containing α-aminoisobutyric acid (Aib) at positions 1 and/or 3, were evaluated for the capacity to stimulate cAMP production in the human osteoblastic cell line SaOS-2. The calculated EC50 values and observed maximum response values are means (±S.E.M.) of data from the number of experiments indicated (n). The basal cAMP level was 6.4 ± 0.8 (n= 4). N.R. indicates that no cAMP response was detected.

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## Example 4:  Circular Dichroism

Circular dichroism (CD) spectroscopy was used to analyze the potential effects that the Aib substitutions had on peptide secondary structure when the peptides were free in solution.  Samples were analyzed in both aqueous phosphate buffer and in phosphate buffer containing 2,2,2-trifloroethanol, an organic solvent which promotes helical structure in oligopeptides, including PTH peptide fragments (Pellegrini, M., *et al., J. Biol. Chem. 273*:10420-10427 (1998); Gronwald, W., *et al., Biol. Chem. Hoppe Seyler 377*:175-186 (1996); Barden, J.A., and Kemp, B.E., *Biochemistry 32*:7126-7132 (1993)).  In phosphate buffer, the helical content of each peptide, estimated from the elipticity observed at 222 nM, was small ($\leq 16\%$); however, [Aib$^{1,3}$,M]PTH(1-14) contained nearly twice as much helix as did [M]PTH(1-14) (16% and 8.1% respectively), as did [Aib$^{1,3}$,M]PTH(1-11), as compared to [M]PTH(1-11) (13% and 7.5% respectively, Table 4).  In 2,2,2-trifluoroethanol, the helical content of each peptide increased; [Aib$^{1,3}$,M]PTH(1-14) and [Aib$^{1,3}$,M]PTH(1-11) exhibited the two highest levels of helical content (56% and 57%, respectively) and were each more helical than their Ala-1,-3-containing counterpart peptides (Figure 6 and Table 4).  The higher helical contents of these two peptides were evident not only from the negative elipticities at 192 nM and 222 nM, but also from the positive elipticities at 192 nM (Fig. 6).  Unmodified PTH(1-11) exhibited the least amount of helical structure (30%), whereas [Aib$^{1,3}$,M]PTH(1-10) was approximately 47% helical (Fig. 6 and Table 4).  These results suggest that the Aib-1,3 modifications increase the helical structure of the N-terminal PTH oligo peptides in the free solution phase.

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Table 4  Helicity in N-terminal PTH peptides

| peptide | $[0]_{222}$obs x $10^{-3}$ | | helical residues (%) | |
|---|---|---|---|---|
| | Phos. | Phos+TFE | Phos. | Phos+TFE |
| PTH(1-14) | -2.6 | -10.6 | 9.1 | 38 |
| [M]PTH(1-14) | -2.3 | -11.9 | 8.1 | 42 |
| Aib$^{1,3}$,M]PTH(1-14) | -4.6 | -15.7 | 16 | 56 |
| PTH(1-11) | -1.8 | -8.4 | 6.5 | 30 |
| [M]PTH(-11) | -2.1 | -9.9 | 7.5 | 35 |
| [Aib$^{1,3}$,M]PTH(1-11) | -3.7 | -16.1 | 13 | 57 |
| [Aib$^{1,3}$,M]PTH(1-10) | -3.2 | -13.1 | 11 | 47 |

Circular dichroism spectra were recorded in either 50 mM phosphate buffer or 50 mM phosphate buffer containing trifluoroethanol (20%) as described in Material and Methods and shown in Figure 6. The mean residue elipticity ($[0]_{222}^{obs}/[0]_{222}^{max}$) x 100; where $[0]_{222}$obs is the mean residue elipticity at 222 nM observed for that peptide and $[0]_{222}$ max is the mean residue elipticity reported for a model helical peptide of 10 amino acids ($-28.1 \times 10^{-3}$; Yang et al. 1986 Methods in Enzymol. 130, 208-269).

### *Example 5: PTH Analogs*

As the first step, Aib was introduced at each position in [M]PTH(1-14). The Aib-scanning data indicated that substitutions at most positions diminished activity.  However, the Aib scan data revealed considerable (8- to 10-fold) improvements in cAMP signaling potency with substitutions at position one and three, and these effects were additive, as [Aib$^{1,3}$,M]PTH(1-14), with an EC$_{50}$ of ~1 nM in HKRK-B28 cells, was 100-fold more potent than [M]PTH(1-14), and at least as potent as PTH(1-34).

Competition binding studies performed with $^{125}$I-[M]PTH(1-21) indicated that most of the Aib substitutions exerted their effects on potency (positive or negative), at least in part, by changing PTH-1 receptor-binding affinity. Thus, the Aib-1 and Aib-3 substitutions each improved the apparent affinity of [M]PTH(1-14) for HKRK-B28 cells by approximately 10-fold, and the combined Aib-1,3 substitution increased affinity by approximately 100-fold.  Likewise, the decreases in cAMP signaling potency caused by most of the other Aib

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substitutions could be explained by decreases in apparent binding affinity, even though, overall, binding affinities were generally 10- to 100-fold weaker than the corresponding cAMP signaling potencies. Two exceptions to this were the peptides substituted at positions 2 and 6, at which signaling potency was comparable with (position 2) or ~10-fold weaker than the corresponding apparent binding affinity. That the substitution of Aib for valine-2 or glutamine-6 impaired signaling activity more than receptor-binding affinity, is consistent with the disproportionate reductions in signaling potency, relative to binding affinity, that occur with substitutions at these positions in PTH(1-34) analogs, and, in fact, result in PTH-1R antagonists (Cohen, F.E., *et al., J. Biol. Chem. 266*:1997-2004 (1991); Gardella, T.J., *et al., J. Biol. Chem. 266*:13141-13146 (1991), Carter 1999 #1180).

The 100-fold increase in cAMP-stimulating potency effect that occurred with the paired Aib-1,3 modification to [M]PTH(1-14) seems consistent with the hypothesis that an $\alpha$-helix in the N-terminal portion of PTH is required for activation of the PTH-1 receptor. The capacity of Aib to stabilize $\alpha$-helical structure in oligopeptides arises from the steric restrictions on the rotations about the N-C° ($\varphi$) and C°-CO ($\psi$) bonds of the Aib residue that are imposed by the two methyl groups symmetrically bonded to its C° atom (Kaul, R., and Balaram, P., *Bioorganic & Medicinal Chemistry 7*:105-117 (1999); Burgess, A.W., and Leach, S.J., *Biopolymers 12*:2599-2605 (1973)). The $\varphi$ and $\psi$ torsion angles about this C° atom are tightly restricted to those that occur in $\alpha$-helices, but the symmetry of the di-alkyl-substituted C° atom of Aib allows for either right-handed or left-handed $\alpha$-helices. If the latter "reversed" configuration occurs in an otherwise right-handed helix, then the Aib residue will, in all probability, induce a turn, and thus terminate the helix (Kaul, R., and Balaram, P., *Bioorganic & Medicinal Chemistry 7*:105-117 (1999); Venkataram Prasad, B.V., *et al., Biopolymers 18*:1635-1646 (1979)). This reversed configuration is rare in peptide structures, relative to the right-handed configuration (Kaul, R., and Balaram, P., *Bioorganic & Medicinal Chemistry 7*:105-117 (1999)), but it nevertheless leaves open the possibility that Aib at the amino-terminus of

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PTH(1-14) enhances potency through some mechanism other than stabilization of an α-helix.

It is also of interest that the most beneficial effects on peptide potency/affinity occurred with Aib substitutions at positions 1 and 3, since none of the structural studies on PTH(1-34) analogs have detected structure N-terminal of residue 3. It may be that Aib at position 1 nucleates helix formation of "down-stream" residues within itself participating in the helix. Alternatively, the modification may induce or stabilize helical structure at the very N-terminus of the peptide which is simply too unstable in the native sequence to be detected by NMR spectroscopy or x-ray crystallography. In any case, the 1000-fold higher cAMP signaling potency exhibited by [$Aib^{1,3}$,$Gln^{10}$,$Har^{11}$]PTH(1-11) as compared to [$Ala^3$,$Gln^{10}$,$Har^{11}$]PTH(1-11) ($EC_{50}$s ~6 nM Vs. 3 μM, respectively, Table 1 and (Shimizu, M., *et al., Endocrinology* (2001) (In Press)) demonstrates that the effects of the Aib substitutions are exerted locally, e.g. within the first 11 amino acids of the peptide.

Direct structural analyses of these analogs, as free peptides, or potentially in complex with the PTH-1 receptor, could provide valuable insights into the ligand structures that allow a ligand to act as an agonist on the PTH-1 receptor. In this regard, the information derived from the data set described herein could be of use in the design of peptide mimetics for the PTH-1 receptor. Approaching this problem from the standpoint of the native PTH peptide sequence is made difficult by the conformational diversity that is possible at each position in the peptide backbone chain. The incorporation of stereochemically constrained amino acids, such as Aib, into the peptide chain, lessens this problem, as it serves to nucleate predictable peptide structures. Thus, the approach can facilitate the *de novo* design of peptide or nonpeptide agonists for the PTH-1 receptor. Given the recently proven utility of PTH(1-34) in treating osteoporosis (Neer, R.M., *et al., N.E.J.M. 344*:1434-1441 (2001)), such agonists should have important medical impact.

At the molecular level, it is presently unclear how the [$Aib^{1,3}$,M]PTH analogs interact with the receptor; nor is this known for any PTH ligand, although

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fairly specific computer models of the interaction with native PTH are now being developed (Jin, L., *et al.*, *J. Biol. Chem.* 275:27238-27244 (2000); Rölz, C., and Mierke, D.F., Biophysical Chemistry (2000) (In Press)). The above described experiments with the truncated PTH-1 receptor, P1R-delNt, provide some insights, as they demonstrate that the enhancing effects of the Aib substitutions at positions 1 and 3 are mediated through the juxtamembrane region (J domain) of the receptor containing the extracellular loops and transmembrane domains. This finding is consistent with the cumulative crosslinking and mutational data on the PTH/PTH-1 receptor interaction, which indicate that residues in the (1-14) domain of PTH interact primarily, if not exclusively, with the receptor's J domain, as opposed to its amino-terminal extracellular domain (N domain) (Bergwitz, C., *et al.*, *J. Biol. Chem.* 271:26469-26472 (1996); Hoare, S.R.J., *et al.*, *J. Biol. Chem* 276:7741-7753 (2001); Behar, V., *et al.*, *J. Biol. Chem.* 275:9-17 (1999); Shimizu, M., *et al.*, *J. Biol. Chem.* 275:19456-19460 (2000); Luck, M.D., *et al.*, *Molecular Endocrinology 13*:670-680 (1999); Shimizu, M., *et al.*, *J. Biol. Chem.* 275:21836-21843 (2000); Carter, P.H., and Gardella, T.J., *Biochim. Biophys. Acta 1538*:290-304 (2001); Gardella, T.J., *et al.*, *Endocrinology 132*:2024-2030 (1993); Bisello, A., et al., J. Biol. Chem. 273:22498-22505 (1998)).

Another important conclusion to derive from our study with P1R-delNt, in which [Aib[1,3],M]PTH(1-14) exhibited low nanomolar potency and full efficacy in cAMP assays and nearly full efficacy in PLC assays, is that the truncated receptor, which lacks nearly all of the N domain, is capable of mounting a sensitive and robust response to a small agonist ligand. The availability of a radioligand that binds to the P1R-delNt, [125]I-[Aib[1,3],M]PTH(1-21), enabled, for the first time, binding studies to be performed on this truncated receptor. Scatchard analysis of our homologous competition binding data yielded Bmax values for P1R-delNt that were not significantly different from those observed for P1R-WT ($1.3 \pm 0.1$ receptors/cell Vs. $1.9 \pm 0.8$ receptors/cell, respectively, $P = 0.3$). Thus, the truncated receptor is well expressed on the surface of COS-7 cells. Not surprisingly, PTH(1-34) failed to inhibit the binding of [125]I-[Aib[1,3],M]PTH(1-21) to P1R-delNt, a result which highlights the importance of the interaction between

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the N domain of the receptor and the C-terminal (15-34) domain of the native peptide in stabilizing the overall hormone-receptor complex. This result also supports the view that the interaction between the amino-terminal portion of PTH and the J domain of the receptor is of very weak affinity (Hoare, S.R.J., *et al., J. Biol. Chem 276*:7741-7753 (2001)). Clearly, the affinity of the interaction can be improved considerably, as the apparent affinity with which [Aib$^{1,3}$,M]PTH(1-14) bound to P1R-delNt ($IC_{50}$ ~1,500 nM) was much greater than that of native PTH(1-14), which failed to inhibit tracer binding. The 50-fold difference that we observed in the affinities with which [Aib$^{1,3}$,M]PTH(1-14) and [Aib$^{1,3}$,M]PTH(1-21) bound to P1R-delNt shows that residues C-terminal of residue 14 (e.g., residues 15-21) contribute binding interactions to the J domain of the receptor. Studies on related analogs suggest that at least some of this effect involves residue 19.

In summary, highly potent PTH(1-14) analogs are obtained by introducing the conformationally constrained amino acid, Aib, at the N-terminus of the peptide. The propensity of Aib to stabilize $\alpha$-helical structure, and the high potency with which the modified analogs activated P1R-delNt, show that the N-terminal portion of PTH is $\alpha$-helical when it is bound to the activation domain of the receptor. The results also establish that the activation domain of the PTH-1R, as defined by P1R-delNt, is fully capable of mediating high affinity and productive interactions with an agonist ligand.

### *Example 6:  PTH(1-34) Derivatives*

We have found that Aib substitutions at positions 1 and 3 in PTH(1-34) ([Tyr34]hPTH(1-34)amide) improve cAMP-stimulating potency on P1R-delNT expressed in COS-7 cells by ~100-fold, relative to unmodified PTH(1-34) (see Table 5 and Figure 7B). The Aib substitutions do not detectably improve potency of PTH(1-34) on the intact wild-type PTH-1 receptor in COS-7 cells (Table 5, and Figure 7A); a result which may be due to the already maximal response mediated by native PTH(1-34) in these cells which express very high levels of the

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intact receptor. In a less sensitive cell system, such as with the delNT receptor, in which nearly the entire amino-terminal extracellular domain of the receptor is deleted, or perhaps in bone cells in animals expressing low levels of endogenous PTH receptors, the effect of Aib-1,3 substitutions on PTH(1-34) potency are significant. Peptides with other, above described modifications (e.g. Gln10, homoArg11, Ala12, Trp14, Arg19) are much more potent than PTH(1-34) in COS-7 cells expressing hP1R-delNT as well. For example, [$Aib^{1,3}$,$Gln^{10}$,$Har^{11}$, $Ala^{12}$,$Trp^{14}$,$Arg^{19}$,$Tyr^{34}$]hPTH(1-34) has an EC50 value of $1.9 \pm 0.6$ nM on P1R-delNT. It is expected that the above described modifications will also be much more potent than PTH(1-34) in other native bone cell systems of low sensitivity.

**Table 5: cAMP Responses of hPTH(1-34) Analogs in COS-7 Cells**

| peptide | EC50(nM) hP1R-WT | EC50(nM) hP1R-delNt |
|---|---|---|
| [$Tyr^{34}$]-hPTH(1-34) | $0.44 \pm 0.02$ | $2,800 \pm 300$ |
| [$Aib^{1,3}$,$Tyr^{34}$]-hPTH(1-34) | $0.67 \pm 0.18$ | $43 \pm 24$ |

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that same can be performed by modifying or changing the invention with a wide and equivalent range of conditions, formulations and other parameters thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned hereinabove are herein incorporated in their entirety and by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1.      A biologically active peptide consisting essentially of the formula selected from:

      (a)      $X_{01}$ValX$_{02}$GluIleGlnLeuMetHisX$_{03}$X$_{04}$X$_{05}$X$_{06}$X$_{07}$ (SEQ ID NO. 1);

      (b)      fragments thereof, containing amino acids 1-11, 1-12 or 1-13;

      (c)      pharmaceutically acceptable salts thereof; or

      (d)      N- or C- derivatives thereof;

wherein:

$X_{01}$ is an α-helix-stabilizing residue, desaminoGly, desaminoSer or desaminoAla;

$X_{02}$ is an α-helix-stabilizing residue, Ala, or Ser;

$X_{03}$ is Ala, Gln or Asn;

$X_{04}$ is Arg, Har or Leu;

$X_{05}$ is an α-helix-stabilizing residue, Ala or Gly;

$X_{06}$ is an α-helix-stabilizing residue or Lys;

$X_{07}$ is an α-helix-stabilizing residue, Trp or His; and

wherein at least one of $X_{01}$, $X_{02}$, $X_{05}$, $X_{06}$ or $X_{07}$ is an α-helix-stabilizing residue.


2.      The peptide of claim 1, wherein said α-helix-stabilizing amino acid is selected from the group consisting of Aib, ACPC (1-aminocyclopropylcarboxylic acid), DEG (diethylglycine) and 1-aminocyclopentanecarboxylic acid.


3.      The peptide of claim 1, wherein said peptide is selected from:

      (a)      AibValSerGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 2);

      (b)      fragments thereof, containing amino acids 1-11, 1-12 or 1-13;

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    (c)     pharmaceutically acceptable salts thereof; or

    (d)     N- or C- derivatives thereof.


    4.     The peptide of claim 1, wherein said peptide is selected from:

    (a)     desaminoAlaValAibGluIleGlnLeuMetHisAsnLeuGlyLys
His (SEQ ID NO. 3);

    (b)     fragments thereof, containing amino acids 1-11, 1-12 or
1-13;

    (c)     pharmaceutically acceptable salts thereof; or

    (d)     N- or C- derivatives thereof.


    5.     The peptide of claim 1, wherein said peptide is selected from:

    (a)     desaminoSerValAibGluIleGlnLeuMetHisAsnLeuGlyLys
His (SEQ ID NO. 4);

    (b)     fragments thereof, containing amino acids 1-11, 1-12 or
1-13;

    (c)     pharmaceutically acceptable salts thereof; or

    (d)     N- or C- derivatives thereof.


    6.     The peptide of claim 1, wherein said peptide is selected from:

    (a)     desaminoGlyValAibGluIleGlnLeuMetHisAsnLeuGlyLys
His (SEQ ID NO. 5);

    (b)     fragments thereof, containing amino acids 1-11, 1-12 or
1-13;

    (c)     pharmaceutically acceptable salts thereof; or

    (d)     N- or C- derivatives thereof.


    7.     The peptide of claim 1, wherein said peptide is selected from:

    (a)     AibValAibGluIleGlnLeuMetHisGlnHarGlyLysTrp (SEQ
ID NO. 6);

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(b)     fragments thereof, containing amino acids 1-11, 1-12 or 1-13;

(c)     pharmaceutically acceptable salts thereof; or

(d)     N- or C- derivatives thereof.


8.      The peptide of claim 1, said peptide selected from:

(a)     AibValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 7);

(b)     fragments thereof, containing amino acids 1-11, 1-12 or 1-13;

(c)     pharmaceutically acceptable salts thereof; or

(d)     N- or C- derivatives thereof.


9.      The peptide of claim 1, said peptide selected from:

(a)     AibValAlaGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 9);

(b)     fragments thereof, containing amino acids 1-11, 1-12 or 1-13;

(c)     pharmaceutically acceptable salts thereof; or

(d)     N- or C- derivatives thereof.


10.     The peptide of claim 1, said peptide selected from:

(a)     AlaValAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 10);

(b)     fragments thereof, containing amino acids 1-11, 1-12 or 1-13;

(c)     pharmaceutically acceptable salts thereof; or

(d)     N- or C- derivatives thereof.

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11.     The peptide of claim 1, said peptide selected from:

      (a)     SerValAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 11);

      (b)     fragments thereof, containing amino acids 1-11, 1-12 or 1-13;

      (c)     pharmaceutically acceptable salts thereof; or

      (d)     N- or C- derivatives thereof.


12.     A biologically active peptide consisting essentially of the formula selected from:

      (a)     AibValAibGluIleGlnLeuNleHisGlnHarAlaLysTrpLeuAla SerValArgArgTyr (SEQ ID NO. 8);

      (b)     fragments thereof, containing amino acids 1-20, 1-19, 1-18, 1-17, 1-16 or 1-15;

      (c)     pharmaceutically acceptable salts thereof; or

      (d)     N- or C- derivatives thereof.


13.     The peptide of claim 1, wherein said peptide is AibValSerGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 2).


14.     The peptide of claim 1, wherein said peptide is desaminoAlaValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 3).


15.     The peptide of claim 1, wherein said peptide is desaminoSerValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 4).


16.     The peptide of claim 1, wherein said peptide is desaminoGlyValAibGluIleGlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 5).


17.     The peptide of claim 1, wherein said peptide is AibValAibGluIle GlnLeuMetHisGlnHarGlyLysTrp (SEQ ID NO. 6).

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18.    The peptide of claim 1, wherein said peptide is AibValAibGluIle GlnLeuMetHisAsnLeuGlyLysHis (SEQ ID NO. 7).

19.    The peptide of claim 12, wherein said peptide is AibValAibGluIle GlnLeuNleHisGlnHarGlyLysTrpLeuAlaSerValArgArgTyr (SEQ ID NO. 8).

20.    The peptide of claim 1, wherein said peptide is AibValAlaGluIle GlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 9).

21.    The peptide of claim 1, wherein said peptide is AlaValAibGluIle GlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 10).

22.    The peptide of claim 1, wherein said peptide is SerValAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO. 11).

23.    The peptide of claim 1 or 12, wherein said peptide is labeled.

24.    The peptide of claim 23, wherein said peptide is labeled with a fluorescent label.

25.    The peptide of claim 23, wherein said peptide is labeled with a chemiluminescent label.

26.    The peptide of claim 23, wherein said peptide is labeled with a bioluminescent label.

27.    The peptide of claim 23, wherein said peptide is labeled with a radioactive label.

28.    The peptide of claim 27, wherein said peptide is labeled with $^{125}$I.

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29.    The peptide of claim 27, wherein said peptide is labeled with $^{99m}Tc$.

30.    A pharmaceutical composition comprising the biologically active peptide of claim 1 or 12, and a pharmaceutically acceptable carrier.

31.    A method for treating mammalian conditions characterized by decreases in bone mass, said method comprising administering to a subject in need thereof an effective bone mass-increasing amount of a biologically active peptide of claim 1 or 12.

32.    A method for treating mammalian conditions characterized by decreases in bone mass, said method comprising administering to a subject in need thereof an effective bone mass-increasing amount of a composition comprising a biologically active peptide of claim 1 or 12 and a pharmaceutically acceptable carrier.

33.    A method for determining rates of bone reformation, bone resorption and/or bone remodeling comprising administering to a patient an effective amount of a peptide of claim 1 or 12 and determining the uptake of said peptide into the bone of said patient.

34.    The method of claim 32, wherein said condition to be treated is osteoporosis.

35.    The method of claim 32, wherein said condition to be treated is old age osteoporosis.

36.    The method of claim 32, wherein said condition to be treated is post-menopausal osteoporosis.

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37.     The method of claim 32, wherein said effective amount of said peptide for increasing bone mass is from about 0.01 μg/kg/day to about 1.0 μg/kg/day.

38.     The method of claim 32, wherein the method of administration is parenteral.

39.     The method of claim 32, wherein the method of administration is subcutaneous.

40.     The method of claim 32, wherein the method of administration is nasal insufflation.

41.     A method of making the peptide of claim 1 or 12, wherein said peptide is synthesized by solid phase synthesis.

42.     The method of making the peptide of claim 1 or 12, wherein said peptide is protected by FMOC.

43.     The peptide of claim 2, wherein said α-helix-stabilizing amino acid is Aib.

44.     A biologically active peptide consisting essentially of the formula selected from:

(a)     $X_{01}$ Val $X_{02}$ GluIleGlnLeu $X_{03}$ His $X_{04} X_{05} X_{06} X_{07} X_{08}$ Leu $X_{09}$ Ser $X_{10} X_{11}$ Arg $X_{12} X_{13}$ Trp LeuArgLysLysLeuGlnAspValHisAsn $X_{14}$ (SEQ ID NO. 19);

(b)     pharmaceutically acceptable salts thereof; or

(c)     N- or C- derivatives thereof;

wherein:

$X_{01}$ is an α-helix-stabilizing residue desaminoGly, desaminoSer or desaminoAla;

$X_{02}$ is an α-helix-stabilizing residue, Ala, or Ser;

$X_{03}$ is Met or Nle;

$X_{04}$ is Ala, Gln or Asn;

$X_{05}$ is Arg, Har or Leu;

$X_{06}$ is an α-helix-stabilizing residue, Ala or Gly;

$X_{07}$ is an α-helix-stabilizing residue or Lys;

$X_{08}$ is an α-helix-stabilizing residue, Trp or His;

$X_{09}$ is Ala or Asn;

$X_{10}$ is Met or Val;

$X_{11}$ is Arg or Glu;

$X_{12}$ is Met or Val;

$X_{13}$ is Gln or Glu;

$X_{14}$ is Tyr or Phe; and

wherein at least one of $X_{01}$, $X_{02}$, $X_{06}$, $X_{07,}$ or $X_{08}$ is an α-helix-stabilizing residue.


45.    The peptide of claim 44, wherein said α-helix-stabilizing amino acid is selected from the group consisting of Aib, ACPC (1-aminocyclopropylcarboxylic acid), DEG (diethylglycine) and 1-aminocyclopentanecarboxylic acid.


46.    The peptide of claim 44, wherein said peptide is selected from:

   (a)    AibValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeu$X_{09}$Ser$X_{10}X_{11}$Arg$X_{12}X_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsn$X_{14}$ (SEQ ID NO. 20);

   (b)    pharmaceutically acceptable salts thereof; or

   (c)    N- or C- derivatives thereof.


47.    The peptide of claim 44, wherein said peptide is selected from:

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    (a)    desaminoAlaValAibGluIleGlnLeuMetHisAsnLeuGlyLys
HisLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$TrpLeuArgLysLysLeuGln
AspValHisAsnX$_{14}$ (SEQ ID NO. 21);

    (b)    pharmaceutically acceptable salts thereof; or

    (c)    N- or C- derivatives thereof.

48.    The peptide of claim 44, wherein said peptide is selected from:

    (a)    desaminoSerValAibGluIleGlnLeuMetHisAsnLeuGlyLys
HisLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$TrpLeuArgLysLysLeuGln
AspValHisAsnX$_{14}$ (SEQ ID NO. 22);

    (b)    pharmaceutically acceptable salts thereof; or

    (c)    N- or C- derivatives thereof.

49.    The peptide of claim 44, wherein said peptide is selected from:

    (a)    desaminoGlyValAibGluIleGlnLeuMetHisAsnLeuGlyLys
HisLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLysLeuGln
AspValHisAsnX$_{14}$ (SEQ ID NO. 23);

    (b)    pharmaceutically acceptable salts thereof; or

    (c)    N- or C- derivatives thereof.

50.    The peptide of claim 44, wherein said peptide is selected from:

    (a)    AibValAibGluIleGlnLeuMetHisGlnHarGlyLysTrpLeuX$_{09}$
SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLysLeuGlnAspValHis
AsnX$_{14}$ (SEQ ID NO. 24);

    (b)    pharmaceutically acceptable salts thereof; or

    (c)    N- or C- derivatives thereof.

51.    The peptide of claim 44, said peptide selected from:

    (a)    AibValAibGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuX$_{09}$
SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLysLeuGlnAspValHis
AsnX$_{14}$ (SEQ ID NO. 25);

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  (b)  pharmaceutically acceptable salts thereof; or

  (c)  N- or C- derivatives thereof.

52.  The peptide of claim 44, said peptide selected from:

  (a)  AibValAlaGluIleGlnLeuMetHisGlnHarAlaLysTrpLeuX$_{09}$
     SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLysLeuGlnAspValHis
     AsnX$_{14}$ (SEQ ID NO. 26);

  (b)  pharmaceutically acceptable salts thereof; or

  (c)  N- or C- derivatives thereof.

53.  The peptide of claim 44, said peptide selected from:

  (a)  AlaValAibGluIleGlnLeuMetHisGlnHarAlaLysTrpLeuX$_{09}$
     SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$TrpLeuArgLysLysLeuGlnAspValHis
     AsnX$_{14}$ (SEQ ID NO. 27);

  (b)  pharmaceutically acceptable salts thereof; or

  (c)  N- or C- derivatives thereof.

54.  The peptide of claim 44, said peptide selected from:

  (a)  SerValAibGluIleGlnLeuMetHisGlnHarAlaLysTrpLeuX$_{09}$
     SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLysLeuGlnAspValHis
     AsnX$_{14}$ (SEQ ID NO. 28);

  (b)  pharmaceutically acceptable salts thereof; or

  (c)  N- or C- derivatives thereof.

55.  A biologically active peptide consisting essentially of the formula
selected from:

  (a)  AibValAibGluIleGlnLeuNleHisGlnHarAlaLysTrpLeuAla
     SerValArgArgX$_{12}$X$_{13}$Trp LeuArgLysLysLeuGlnAspValHis
     AsnX$_{14}$ (SEQ ID NO. 29);

  (b)  pharmaceutically acceptable salts thereof; or

  (c)  N- or C- derivatives thereof;

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wherein

$X_{12}$ is Met or Val;

$X_{13}$ is Gln or Glu; and

$X_{14}$ is Tyr or Phe.

56.    The peptide of claim 44, wherein said peptide is AibValSerGluIle GlnLeuMetHisAsnLeuGlyLysHisLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$TrpLeuArgLysLys LeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 20).

57.    The peptide of claim 44, wherein said peptide is desamino-AlaValAibGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 21).

58.    The peptide of claim 44, wherein said peptide is desamino-SerValAibGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLysLeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 22).

59.    The peptide of claim 44, wherein said peptide is desamino-GlyValAibGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$ Trp LeuArgLysLysLeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 23).

60.    The peptide of claim 44, wherein said peptide is AibValAibGluIle GlnLeuMetHisGlnHarGlyLysTrpLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$TrpLeuArgLysLys LeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 24).

61.    The peptide of claim 44, wherein said peptide is AibValAibGluIle GlnLeuMetHisAsnLeuGlyLysHisLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLys LeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 25).

62.    The peptide of claim 44, wherein said peptide is AibValAlaGluIle GlnLeuMetHisGlnHarAlaLysTrpLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLys

LeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 26).


63.     The peptide of claim 44, wherein said peptide is AlaValAibGluIle GlnLeuMetHisGlnHarAlaLysTrpLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLys LeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 27).


64.     The peptide of claim 44, wherein said peptide is SerValAibGluIle GlnLeuMetHisGlnHarAlaLysTrpLeuX$_{09}$SerX$_{10}$X$_{11}$ArgX$_{12}$X$_{13}$Trp LeuArgLysLys LeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 28).


65.     The peptide of claim 55, wherein said peptide is AibValAibGluIle GlnLeuNleHisGlnHarGlyLysTrpLeuAlaSerValArgArgX$_{12}$X$_{13}$Trp LeuArgLysLys LeuGlnAspValHisAsnX$_{14}$ (SEQ ID NO. 29).


66.     The peptide of claim 44 or 55, wherein said peptide is labeled.


67.     The peptide of claim 66, wherein said peptide is labeled with a fluorescent label.


68.     The peptide of claim 66, wherein said peptide is labeled with a chemiluminescent label.


69.     The peptide of claim 66, wherein said peptide is labeled with a bioluminescent label.


70.     The peptide of claim 66, wherein said peptide is labeled with a radioactive label.


71.     The peptide of claim 70, wherein said peptide is labeled with $^{125}$I.

-56-

72.     The peptide of claim 70, wherein said peptide is labeled with $^{99m}Tc$.

73.     A pharmaceutical composition comprising the biologically active peptide of claim 44 or 55, and a pharmaceutically acceptable carrier.

74.     A method for treating mammalian conditions characterized by decreases in bone mass, said method comprising administering to a subject in need thereof an effective bone mass-increasing amount of a biologically active peptide of claim 44 or 55.

75.     A method for treating mammalian conditions characterized by decreases in bone mass, said method comprising administering to a subject in need thereof an effective bone mass-increasing amount of a composition comprising a biologically active peptide of claim 44 or 55 and a pharmaceutically acceptable carrier.

76.     A method for determining rates of bone reformation, bone resorption and/or bone remodeling comprising administering to a patient an effective amount of a peptide of claim 44 or 55 and determining the uptake of said peptide into the bone of said patient.

77.     The method of claim 75, wherein said condition to be treated is osteoporosis.

78.     The method of claim 75, wherein said condition to be treated is old age osteoporosis.

79.     The method of claim 75, wherein said condition to be treated is post-menopausal osteoporosis.

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80.    The method of claim 75, wherein said effective amount of said peptide for increasing bone mass is from about 0.01 µg/kg/day to about 1.0 µg/kg/day.

81.    The method of claim 75, wherein the method of administration is parenteral.

82.    The method of claim 75, wherein the method of administration is subcutaneous.

83.    The method of claim 75, wherein the method of administration is nasal insufflation.

84.    A method of making the peptide of claim 44 or 55, wherein said peptide is synthesized by solid phase synthesis.

85.    The method of making the peptide of claim 44 or 55, wherein said peptide is protected by FMOC.

86.    The peptide of claim 45, wherein said α-helix-stabilizing amino acid is Aib.

Figure 1 (B28)
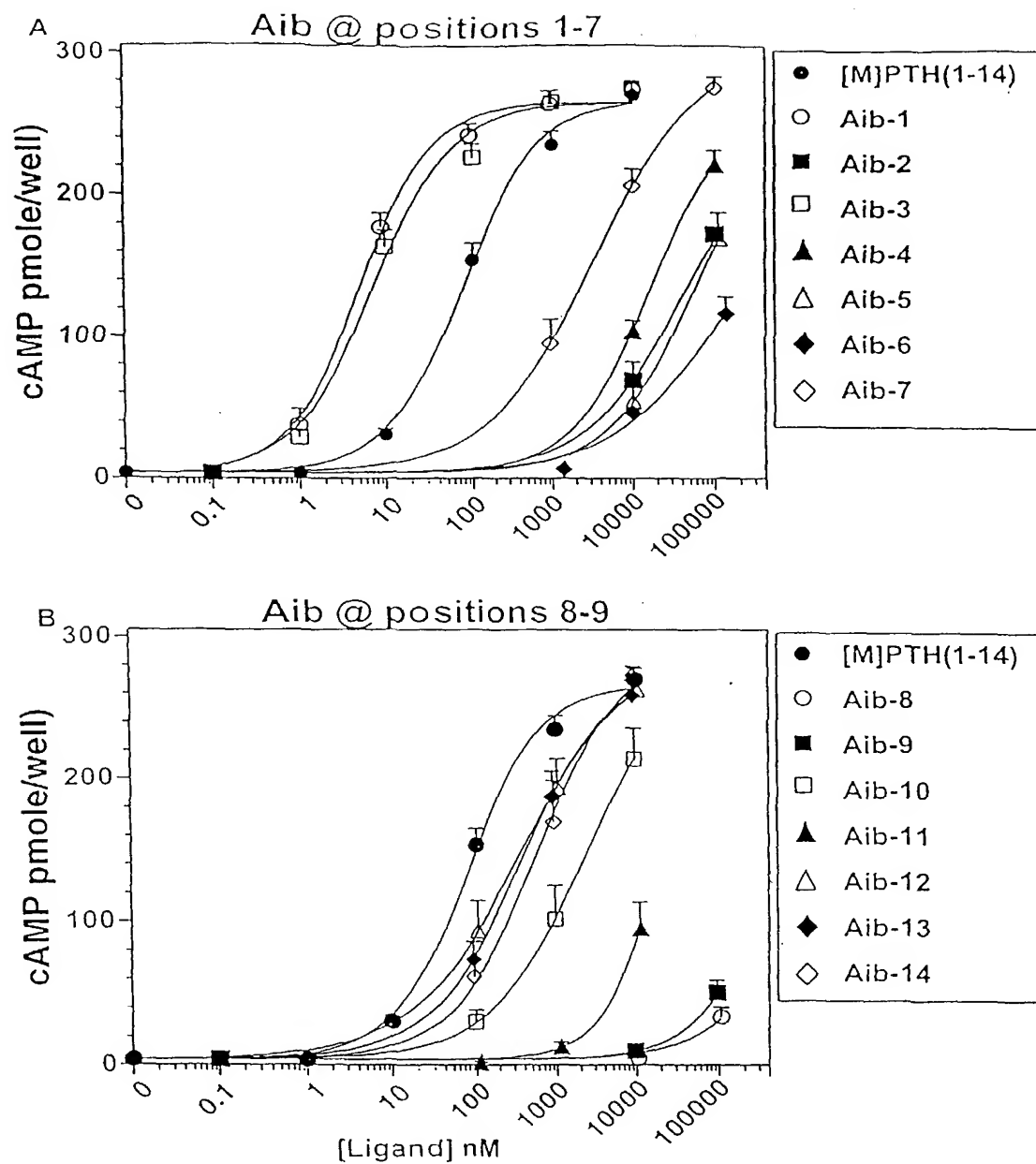
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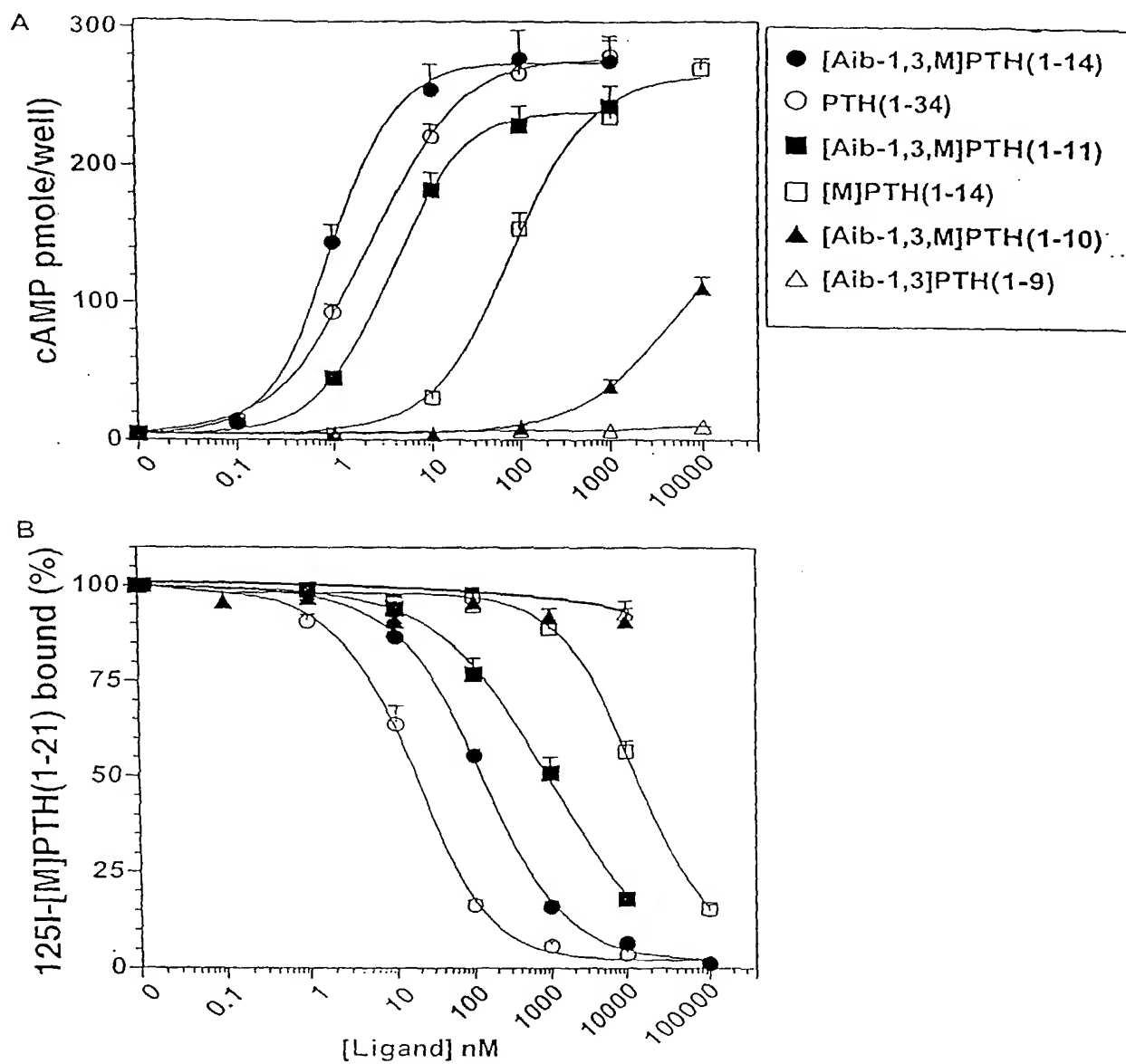
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( delNt)
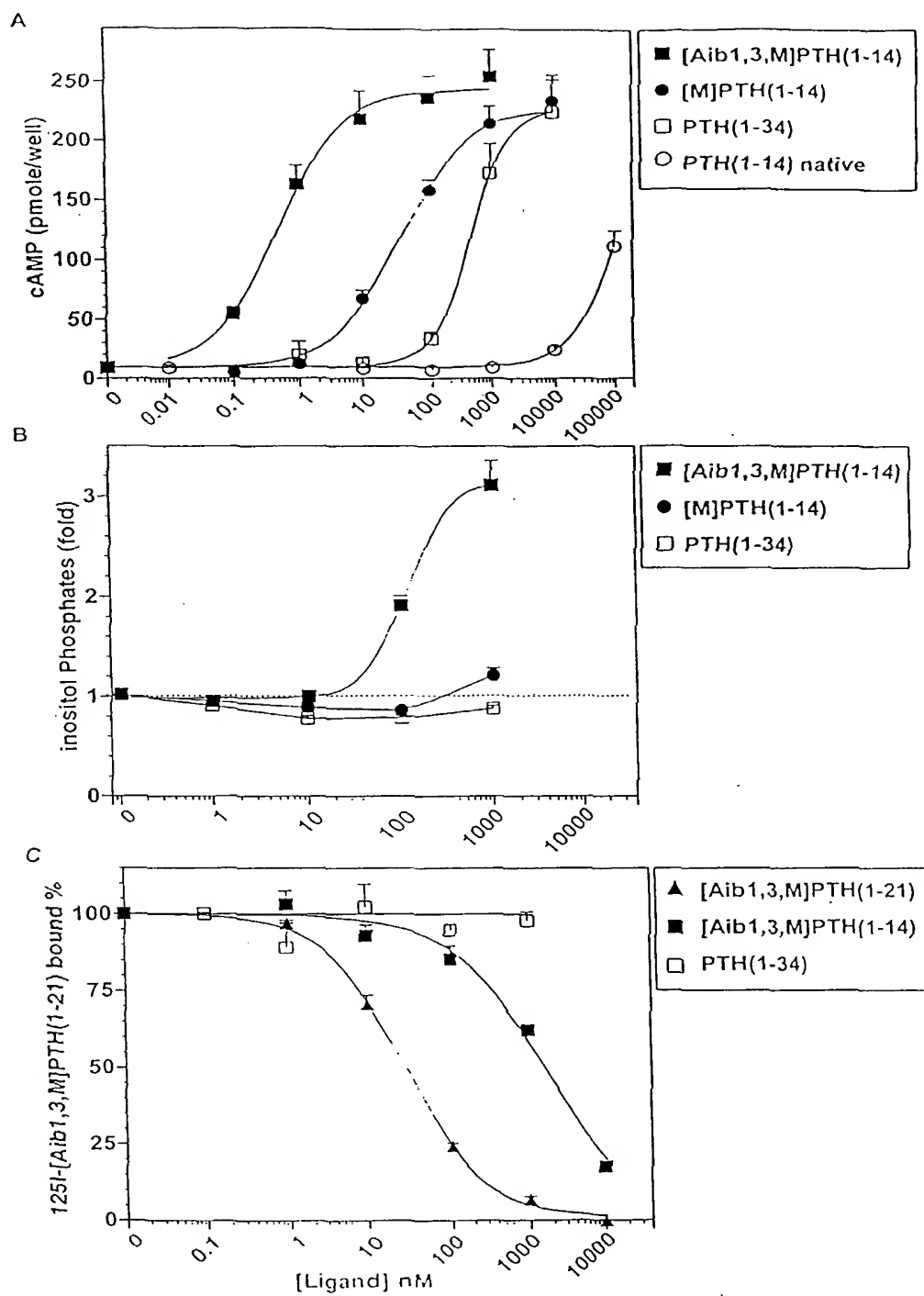
Figure 4
(SaOS-2)

Figure 5
(metatarsal)



A    Vehicle

B    PTH(1-34) 0.1uM

C    [Aib1,3,M]PTH(1-14) 1 uM

D    PTH(1-14) 2uM

Figure 6

Figure 7



**A**

hP1R-WT

**B**

hP1R-delNt

Legend:

■ [Y34]hPTH(1-34)

● [Aib1,3,Y34]hPTH(1-34)
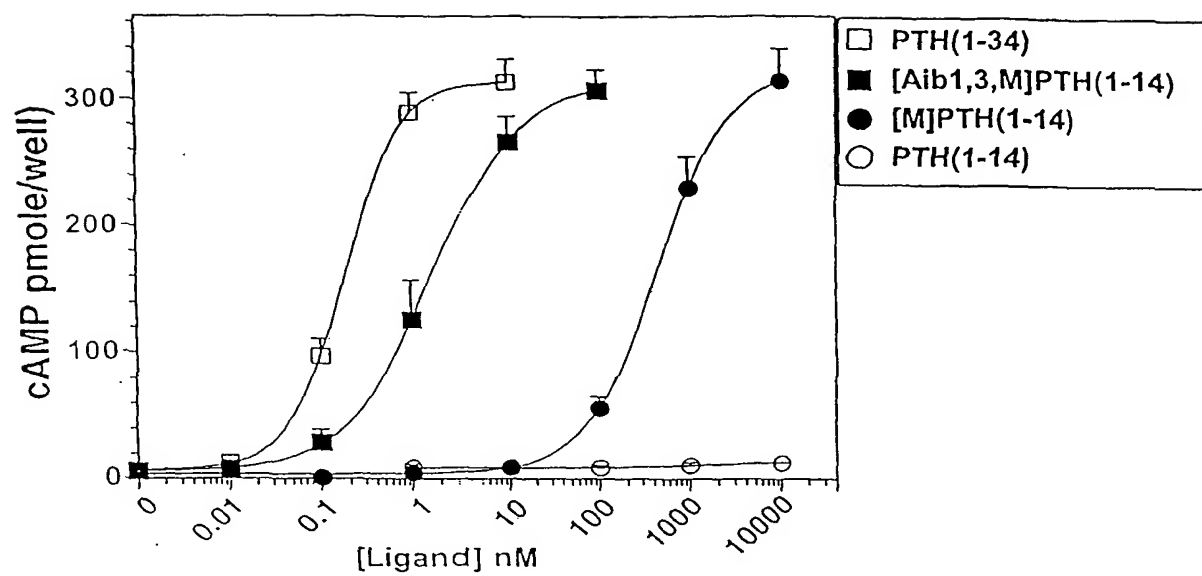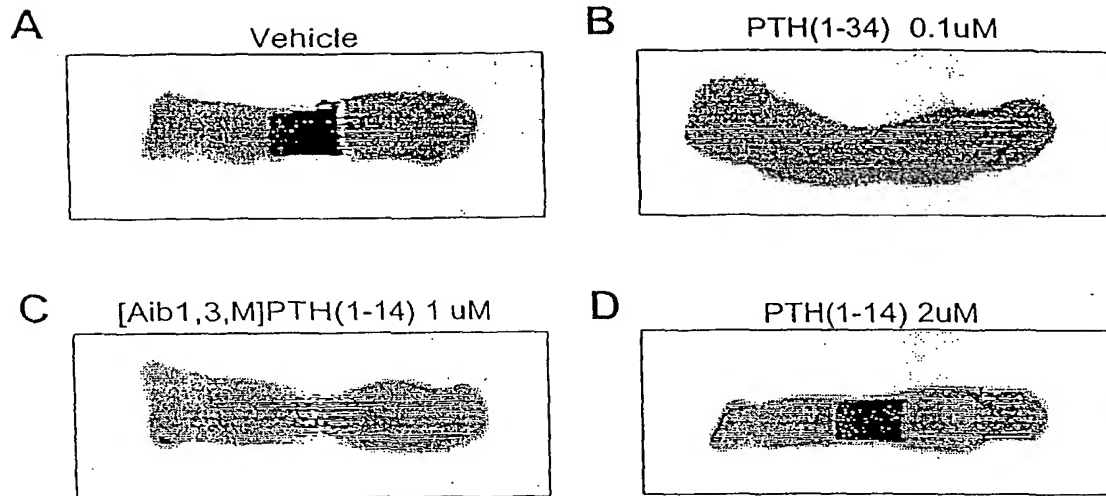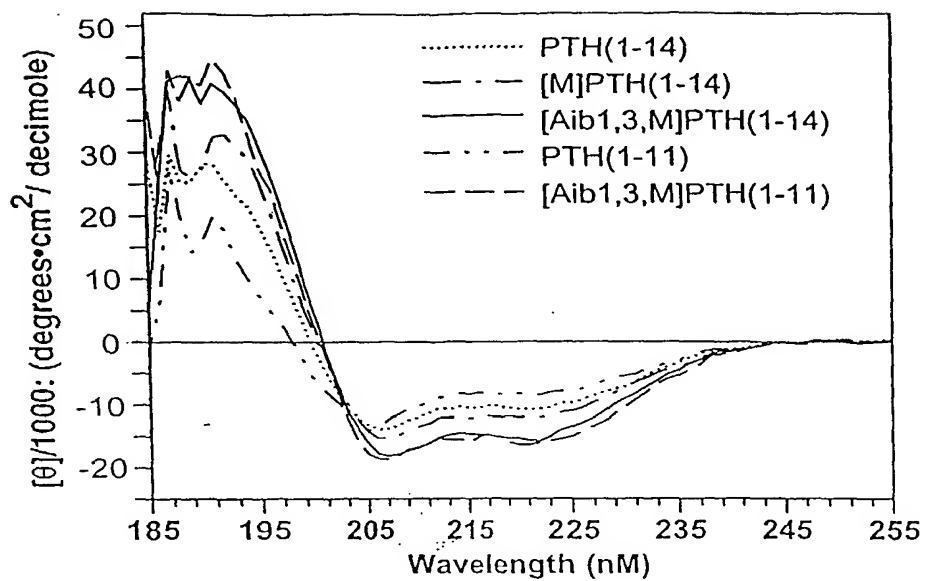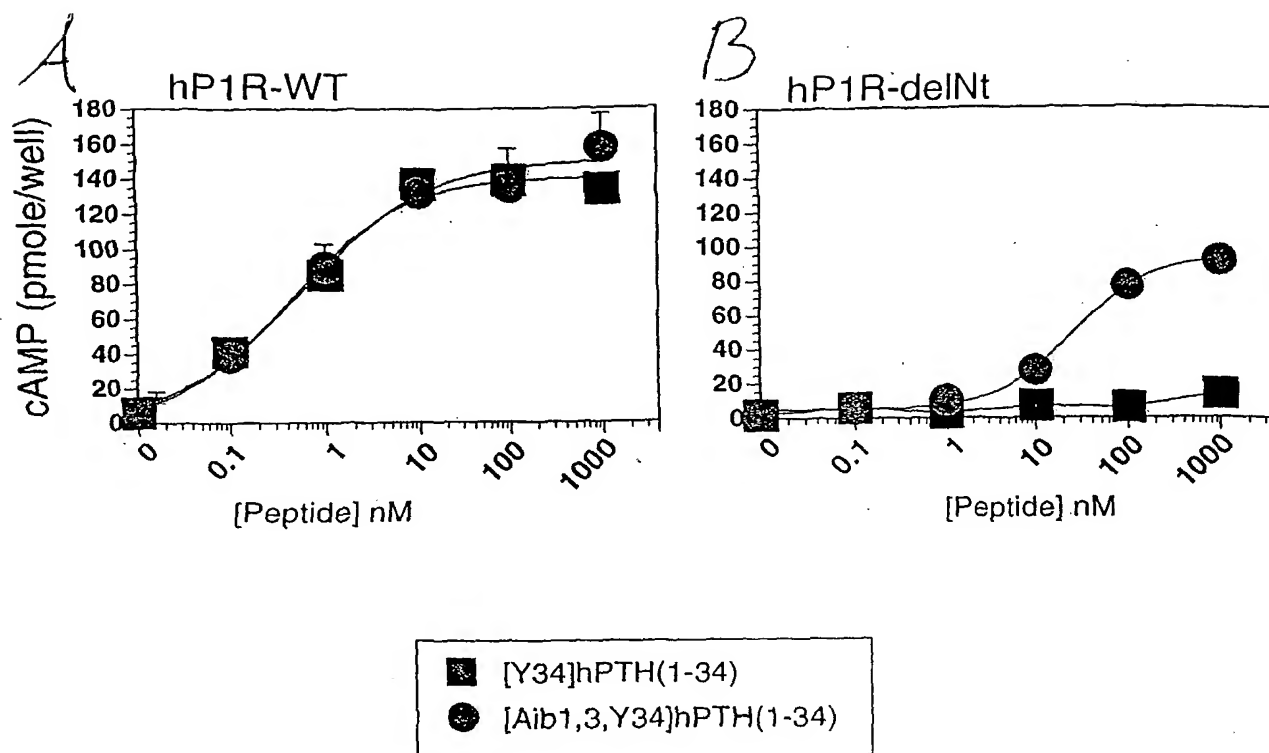
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&lt;223&gt;  Gln or Glu


&lt;220&gt;

&lt;221&gt;  VARIANT

&lt;222&gt;  (34)..(34)

&lt;223&gt;  Tyr or Phe


&lt;400&gt;  23

Xaa Val Xaa Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Xaa
1                   5                  10                  15

Ser Xaa Xaa Arg Xaa Xaa Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20              25                  30

Asn Xaa


&lt;210&gt;  24

&lt;211&gt;  34

&lt;212&gt;  PRT

&lt;213&gt;  Artificial


&lt;220&gt;

&lt;223&gt;  Derivative of PTH Polypeptide

&lt;220&gt;

&lt;221&gt;  MOD_RES

&lt;222&gt;  (1)..(1)

&lt;223&gt;  Aib


&lt;220&gt;

&lt;221&gt;  MOD_RES

&lt;222&gt;  (3)..(3)

&lt;223&gt;  Aib

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<221>   VARIANT

<222>   (16)..(16)

<223>   Ala or Asn


<220>

<221>   VARIANT

<222>   (18)..(18)

<223>   Met or Val


<220>

<221>   VARIANT

<222>   (19)..(19)

<223>   Arg or Glu


<220>

<221>   VARIANT

<222>   (21)..(21)

<223>   Met or Val


<220>

<221>   VARIANT

<222>   (22)..(22)

<223>   Gln or Glu


<220>

<221>   VARIANT

<222>   (34)..(34)

<223>   Tyr or Phe


<220>
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<221> MOD_RES

<222> (11)..(11)

<223> Homoarginine


<400> 24

Xaa Val Xaa Glu Ile Gln Leu Met His Gln Xaa Gly Lys Trp Leu Xaa
1               5                   10                  15

Ser Xaa Xaa Arg Xaa Xaa Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20              25                  30

Asn Xaa


<210> 25

<211> 34

<212> PRT

<213> Artificial


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<223> Derivative of PTH Polypeptide

<220>

<221> MOD_RES

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<223> Aib


<220>

<221> MOD_RES

<222> (3)..(3)

<223> Aib


<220>

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<222> (16)..(16)

<223> Ala or Asn

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<222> (18)..(18)

<223> Met or Val

<220>

<221> VARIANT

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<223> Arg or Glu

<220>

<221> VARIANT

<222> (21)..(21)

<223> Met or Val

<220>

<221> VARIANT

<222> (22)..(22)

<223> Gln or Glu

<220>

<221> VARIANT

<222> (34)..(34)

<223> Tyr or Phe

<400> 25

Xaa Val Xaa Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Xaa
1               5                   10                  15

Ser Xaa Xaa Arg Xaa Xaa Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20                  25                  30

Asn Xaa


<210>  26

<211>  34

<212>  PRT

<213>  Artificial


<220>

<223>  Derivative of PTH Polypeptide

<220>

<221>  MOD_RES

<222>  (1)..(1)

<223>  Aib


<220>

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<223>  Homoarginine


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<223>  Ala or Asn


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<223>  Tyr or Phe


<400>  26

Xaa Val Ala Glu Ile Gln Leu Met His Gln Xaa Ala Lys Trp Leu Xaa
1               5                   10                  15


Ser Xaa Xaa Arg Xaa Xaa Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20                  25                  30


Asn Xaa


<210>  27
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<223>    Derivative of PTH Polypeptide

<220>

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<400>   27

Ala Val Xaa Glu Ile Gln Leu Met His Gln Xaa Ala Lys Trp Leu Xaa
1               5                   10                  15


Ser Xaa Xaa Arg Xaa Xaa Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20                  25                  30


Asn Xaa


<210>   28

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<220>

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<222>  (16)..(16)

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<223>  Arg or Glu


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<223>  Met or Val


<220>

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<222>  (22)..(22)

<223>  Gln or Glu


<220>

<221>  VARIANT

<222>  (34)..(34)
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&lt;223&gt;  Tyr or Phe


&lt;400&gt;  28

Ser Val Xaa Glu Ile Gln Leu Met His Gln Xaa Ala Lys Trp Leu Xaa
1               5                   10                  15


Ser Xaa Xaa Arg Xaa Xaa Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20              25                  30


Asn Xaa


&lt;210&gt;  29

&lt;211&gt;  34

&lt;212&gt;  PRT

&lt;213&gt;  Artificial


&lt;220&gt;

&lt;223&gt;  Derivative of PTH Polypeptide

&lt;220&gt;

&lt;221&gt;  MOD_RES

&lt;222&gt;  (1)..(1)

&lt;223&gt;  Aib


&lt;220&gt;

&lt;221&gt;  MOD_RES

&lt;222&gt;  (3)..(3)

&lt;223&gt;  Aib


&lt;220&gt;

&lt;221&gt;  MOD_RES

&lt;222&gt;  (8)..(8)

&lt;223&gt;  Nle

<220>

<221> MOD_RES

<222> (11)..(11)

<223> Homoarginine


<220>

<221> VARIANT

<222> (21)..(21)

<223> Met or Val


<220>

<221> VARIANT

<222> (22)..(22)

<223> Gln or Glu


<220>

<221> VARIANT

<222> (34)..(34)

<223> Tyr or Phe


<400> 29

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Xaa Val Xaa Glu Ile Gln Leu Xaa His Gln Xaa Ala Lys Trp Leu Ala
1               5                   10                  15


Ser Val Arg Arg Xaa Xaa Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20                  25                  30


Asn Xaa
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<210> 30

<211> 34

<212> PRT

<213> Rattus sp.

<400> 30

Ala Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Ala
1               5                   10                  15

Ser Val Glu Arg Met Gln Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20                  25                  30

Asn Phe


<210> 31

<211> 34

<212> PRT

<213> Homo sapiens


<400> 31

Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn
1               5                   10                  15

Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20                  25                  30

Asn Phe